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DANIEL I. ARNON, *Editor*
University of California

LEONARD MACHLIS, *Associate Editor*
University of California

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THE ANNUAL REVIEW AND THE BOUNDARIES OF PLANT PHYSIOLOGY

With the appearance of volume 6 of the *Annual Review of Plant Physiology*, my current term as Editor comes to an end. It seems fitting on this occasion to restate the objectives of the *Review* and to attempt a personal appraisal of its accomplishments from the first tentative plans eight years ago to the present solidity of the six volumes on the library shelf. I shall try to combine my appraisal of the *Review* with some thoughts on the shifting boundaries of plant physiology as a field of inquiry.

The *Annual Review* has been launched in the hope that it would help to provide integration and encourage synthesis in the broad field of plant physiology, and thus aid in knitting closer ties between physiologists active in diverse fields of research. The multiplicity and diversity of publications in plant physiology has threatened to create an ever-increasing gap between physiologists engaged in fundamental research and those in agricultural disciplines. Our publication has undertaken to bridge this gap by inviting critical reviews by workers of distinction in their respective areas of activity. The selection of topics has been wide and has purposely included borderline areas. The intent has been to cover the whole field of plant physiology not in any one year but within a period of several years. The contents of each volume have been built around a core of annual reviews in areas of great research activity such as inorganic nutrition of plants, photosynthesis, and growth substances. This core of annual articles has been supplemented each year by subjects in which the extent of current research and publication justified reviews only at less frequent intervals.

The special realities of research in plant physiology were responsible for departures from the editorial pattern of the parent publication, the *Annual Review of Biochemistry*. The long periods required for growing higher plants restrict the volume of published output in many areas of plant physiology and often make it difficult to adhere to calendar regularity in reviewing a given topic. The abundance of borderline subjects, the shifting emphasis in research, and the individual competence and preference of reviewers for a selected aspect of a subject have made it impossible to retain a continuity of titles even in those topics which were reviewed each year. Thus, in the six volumes, topics relating to inorganic nutrition of plants appeared under nineteen different titles and those on carbon assimilation under ten. This flexibility, however, has provided for a more detailed and incisive coverage in keeping with the growth of a subject.

From its very beginning the *Annual Review* has striven to present a critical analysis of the literature in a cohesive manner relating current developments to past progress. The editorial suggestions to authors have stressed this, above all, as the desired dominant note in the reviews. In covering the literature authors have been encouraged to shun encyclopedic completeness at the expense of critical evaluation of selected material.

The policy of rotating reviewers has been relied upon to bring, over a period of several years, balance and completeness of coverage in individual areas of plant physiology.

The cordial reception given to the *Review* by plant physiologists from all quarters was a tribute to our authors whose articles fulfilled the expectations of their colleagues. In the first years of its existence the *Review* has found what is hoped will be a permanent place of usefulness in the library of the investigator, teacher, and advanced student. It is a pleasant task to acknowledge here the many expressions of appreciation from our colleagues for the aid which they have received from the *Review* in helping them enter or in guiding the entry of their graduate students into a new area of research. The *Review* also seems to be increasingly relied upon in the advanced teaching of plant physiology. Despite its youth as a publication, the *Review* already fulfills a useful bibliographical function in the scientific literature, as judged by the frequency with which it is cited.

The plant physiologist who is surveying contemporary activity in his science sees an increasing reliance on biochemical and biophysical approaches in the solution of old physiological problems. It is not surprising, therefore, that the question is sometimes asked whether plant physiology, or all physiology for that matter, is to retain its identity as a science, or is to be swallowed up by biochemistry? Should plant physiology rededicate itself as plant biochemistry and sail under this banner from now on? Should our publication change its name, as some of our colleagues have suggested, to the *Annual Review of Plant Biochemistry*?

In answering these questions it might be well to recall the truism that all divisions of science are artificial and are instituted largely for purposes of human convenience or, perhaps more precisely, of academic administration. The most vigorous and stimulating growth of a science is usually at its boundaries. With the advent of modern knowledge of atomic structure the boundary between physics and chemistry has become so blurred that in Berkeley, for example, the answer, not wholly in jest, to the question what is physics and what is chemistry is often that "if the work was done in the Chemistry Building it is chemistry and if it was done in the Physics Building it is physics." As science advances, the artificial fences erected between disciplines become weaker and weaker and it becomes a futile exercise in semantics to try to determine whether one is a biochemical physiologist, a physiological chemist, a plain physiologist, or a plain biochemist, whatever these terms were once intended to signify.

Inherently, there is nothing permanent about plant physiology or any other branch of science. Tradition apart, the claim for a continued identity or a possible realignment of a discipline must be judged by the pragmatic test whether a particular form of scientific organization is best designed for the advancement of knowledge in a particular area. The distinguishing mark of plant physiology as a science is that it seeks to understand the vital processes of the entire plant. There will be a continuing need for the synthesis

of knowledge gained from studies at the cellular, subcellular, and molecular levels into an understanding of a complete physiological process and the functioning of a whole organism. In seeking this objective plant physiology must use whatever tools and techniques will advance its purpose.

The use of biochemical and biophysical techniques today in physiological laboratories is not different in essence from the addition of chemical laboratories to greenhouses, which must have startled the botanists of yesterday. To a physiologist, however, a biochemical approach is only *one* of several which can advance his science in its present stage of development. To a biochemist, on the other hand, plants are only *one* source of biologica lmaterial which he uses to interpret life phenomena in molecular terms. By retaining his identity, the physiologist preserves a traditional freedom of choosing a technique, a method of attack which will best suit his experimental objective. He is free to oscillate between a rigorous biochemical experiment concerned with the stoichiometry of an isolated enzyme reaction and a growth experiment in which the critical data do not go beyond dry weight determinations. He must accord equal dignity and recognition to all techniques which will advance his understanding of plant life. He must recognize that there is an inevitable unevenness in the development of different branches of physiology.

The physiologist does not set limits to the understanding of life processes, which he is seeking. He is, or should be, just as interested as the biochemist in striving to interpret cellular activity in the most fundamental terms possible. He must agree with the biochemist today to seek an understanding of physiological processes in molecular terms. He must be prepared tomorrow to seek with the physicist an understanding of life phenomena in atomic or subatomic terms. The discussion as to whether physiology is being superseded by biochemistry is reminiscent of the views of some physicists that chemistry is only an empirical method of studying facts which could be discovered from the basic principles of physics. To quote from the recent book on the philosophy of science by Herbert Dingle: "Chemistry has no place in the strict scientific scheme. . . . The periodic table, originally a product of chemical research, is a product also of spectroscopic research, and with this difference, that instead of showing in each place a chemical symbol and an atomic weight, the spectroscopic table shows a configuration of electric charges which, when fully understood, will undoubtedly prescribe all the varieties of chemical combination that are possible. The whole of chemistry may, therefore, so far as final results go, be regarded as a superfluous study."

But we have not yet attained this scientific millennium. Neither Professor Dingle, nor anyone else, is seriously proposing that chemistry or physiology abandon its labours and wait for future shortcuts derived from first principles. The continuing development of plant physiology in providing fresher and deeper insights into the basic problems of plant growth and metabolism is essential not only to the fundamental aim of all science, that of broadening the understanding of the world about us, but also to the advancement of

human welfare. Plant physiology holds the key to the world's food supply. It is destined not only to widen the scientific base on which agriculture now rests, but also to provide new techniques and approaches transcending present-day agriculture for solving the world's food problems in the future.

I think that I am expressing a justifiable hope that the *Annual Review of Plant Physiology* will play an important role in the future growth of plant physiology. With the multiplicity and complexity of advances in various branches of our science, there will be a growing need for critical evaluation and synthesis. In performing this function, the *Annual Review* can lay claim to having already established itself as a hardy perennial.

In taking leave of the *Review*, I wish to express my appreciation to the Board of Directors of Annual Reviews, Inc., and its Managing Editor, Dr. J. Murray Luck, for their readiness to launch a new publication and to underwrite the attendant financial obligations. To the new Editor, Dr. L. R. Blinks, go my cordial wishes for success in his new duties. My profound thanks are due to my editorial colleagues, Dr. Machlis and the members, past and present, of the Editorial Committee, who have given me help and counsel in generous measure. I am indebted to Beryl Daniel, and the editorial assistants who preceded her, for devoted and helpful assistance. I also wish to thank our printers, the George Banta Publishing Company for their unfailing cooperation over the years.

DANIEL I. ARNON

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MECHANISMS OF ABSORPTION, TRANSPORT, ACCUMULATION, AND SECRETION OF IONS[1]

By H. Lundegårdh
Penningby, Sweden

Transport of ions involving osmotic work is more characteristic of plants than of animals, a fact obviously related to the need for high osmotic values in most living cells and for osmotic mechanisms of sap movement. The whole complex of problems pertinent to the question of active transport has been extensively debated in a number of comprehensive reviews (1 to 6, 12). In a difficult field such as this, there is danger of overemphasizing theoretical speculation at the expense of experimental work. A few papers are exclusively devoted to thermodynamic speculations (3, 7), and in this area one frequently proceeds without knowledge of the vast already existing experimental material which must be adequately evaluated before it may be described as real progress. Conway (8) has treated the general problem of a "redox pump" for ions from a thermodynamic point of view, but his scheme shows no salient features which may be distinguished from the theory of anion respiration. The author presented a review of the anion respiration to the symposium on active transport of ions in Bangor, England in 1952 (12). The substance of the present article is much the same, but the discussion of the more important literature has been extended.

Passive and Active Transport

If a substance is present in two concentrations, c_o lower than c_1, separated by a permeable membrane, ions will spontaneously or passively go from c_1 to c_o. Some energy is lost during this downhill process. A movement of ions from c_o to c_1 concomitantly requires the coupling to an exergonic process delivering the osmotic energy required to push the ions uphill.

Whereas we know fairly well that active transport of ions is going on in cells and tissues, at the expense of metabolic energy, the role of passive diffusion is still incompletely understood. Diffusion is a very slow-working process where macroscopic dimensions are concerned. It certainly is infinitesimal in the long-line transport through the stele. But its influence increases tremendously when we consider submicroscopic dimensions. It can be calculated that a particle of an average diameter 10^{-8} cm., e.g., a low hydrated inorganic cation, moves by diffusion a distance of 1.15 μ in 0.2 seconds (9). This distance corresponds to the thickness of the cellulose membrane of many parenchyma cells and it far exceeds that of the plasma membrane. We arrive at the conclusion that transport by diffusion may be of considerable influence in the thin layer of protoplasma coating outgrown cells. More ac-

[1] The survey of the literature pertaining to this review was concluded in October, 1954.

curate calculation of the magnitude of diffusion is hampered by the fact that the coefficient of viscosity varies in the protoplasm. The retarding effect of hydration of ions is well-known. The author once found (9) that divalent cations move much more slowly through the cell wall than do monovalent ones. Also, adsorption in the membrane can act as a brake. Similarly, an interaction with ion carriers (see below) severely affects the velocity of diffusion. Protoplasmic streaming may, in a general way, aid a passive transport, but here solid facts are scanty.

Bogen (10) defines permeability as a structurally regulated diffusion in the direction of the concentration gradient. The movement of the ions is, as a rule, retarded by the complicated micellar structure of a plasma membrane, but cases may be found, in which an acceleration of the penetration is brought about by transitorial linkage to exergonic processes. Such cases have been extensively discussed by Danielli (75) who speaks of enhanced or "facilitated diffusion." In the authors opinion, this conception is conflicting, because diffusion is by definition passive and every influence speeding up the passage must be called *active transport*, even if the substance moves in the direction of the concentration gradient.

Ion Carriers

Several earlier investigators [see (3, 11, 12, 18)] have developed the concept of ion carriers. It is accepted by practically all recent investigators of active transport of ions and also of organic substances (5, 15). Ion carriers are thought of as large and practically nondiffusable ions scattered in the protoplasm and its membranes. The concept tallies with the ampholytic properties of the protoplasm. The absorption of ions from the surrounding medium is represented as a reversible reaction between ion and carrier, at equivalent absorption following the formula

$$M^+ + A^- + R^- + R^+ \rightleftarrows M^+R^- + R^+A^-$$

if R^- is the cation carrier and R^+ the anion carrier. It is calculated that the reaction moves to the right at the surface of the cell and to the left somewhere in the interior.

If ion carriers are defined as points of adsorption they are most conveniently studied on the basis of ion exchange. The author introduced two lines of investigation, viz., by quantitative determination of ions adsorbed and ions exuded by the tissue and by measurements of the surface potentials (9, 11, 19, 20). Roots of cereals (wheat, rye, barley), both intact and isolated from seedlings, served as experimental material. Metallic cations are exchanged between the medium and living roots according to the general principles of ion exchange (9) and it was later shown that these processes proceed independently of respiration (21).

The capacity of the living cells to bind ions depends, of course, on the volume concentration of carrier ions R^- and R^+. Comparatively large quantities of metallic cations, primarily $^*K^+$ and Ca^{++}, are going out from roots

exposed to a dilute acid (ca. pH 3.3) in exchange for H^+ (22), whereas only little anions are exuded in more alkaline solutions, facts indicating a quotient (cR^-/cR^+) considerably above 1. Starting from somewhat different viewpoints, Hope & Robertson (24) calculated the concentration of cation carriers in bean root tips to $6.10^{-3}-10^{-2}$ *M*. Vervelde (25) arrives at the high value 3.10^{-2} *M*. These values are of a similar magnitude to those which were calculated earlier for the plasma membrane by the author (9, 11), viz., 10^{-3} to 10^{-2} *M* for roots of cereals, tomato, and flax. In wheat roots the quotient R^-/R^+ amounts to about 100 and it can be concluded from the predominating acid dissociation of the protoplasm (i.e., according to Hope & Robertson at pH 3–4) that values of this magnitude are attained also in the bulk of the protoplasm.

The entry of the ions into the bulk of the protoplasm was at an early date (11) estimated as a successively proceeding ion exchange between carriers in the plasma membrane with carriers in the bulk of the protoplasm, and this scheme is now accepted by most recent investigators. Hope & Robertson (24) claim a cooperation of diffusion and streaming, too, in this process. There is obviously no real difference between this scheme and Arisz' (15, 16) conception of "transport through plasmatic binding." A transport mediated by carriers will always be widely dependent upon metabolic processes which maintain the activity of the large carrier ions (proteins, nucleotides, phosphatides, etc.). This has been emphasized by Arisz (15) who found that inhibition of the respiration renders transport through the protoplasm of *Vallisneria* leaves impossible [the same result was found in *Drosera* tentacles (16)]. In other cases, e.g., in roots of cereals, salts may be transported into the stele at a reduced speed if the cytochrome system is inhibited by cyanide or the glycolysis inhibited by fluoride (21, 26). A strong inhibitor of salt transport is dinitrophenol (DNP),[2] according to Butler (27). Transport by means of passive diffusion is, of course, independent of the respiration and some plasmatic carriers may also resist anaerobiosis. These phenomena are certainly both species-specific and tissue-specific and a generalization of the results should be avoided.

The term "apparent free space" (A.F.S.)[2] has been introduced as an expression of the "proportion of the tissue which appears to reach the external concentration during the initial uptake" (23, 24). Values calculated from this viewpoint are rather high, amounting to 15 per cent (27) or 8 per cent (28) of the total volume of wheat roots.

It is here assumed that a diffusion equilibrium is attained between the medium and A.F.S. But diffusion obviously plays a more subordinate role as compared with ion exchange. Ion exchange commonly follows the rules of adsorption, according to which the quotient $\frac{\text{internal concentration}}{\text{external concentration}}$ is high

[2] The following abbreviations will be used: DNP, dinitrophenol; AFS, apparent free space; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; dwr, distilled water respiration.

at a lower concentration and low at high external concentrations. This would mean a fairly elastic value of A.F.S., a fact also recognized by the inventors of the term. If most of the absorbed ions are adsorbed to carriers the expression "exchange capacity" would be more adequate. The free space ,however, undoubtedly includes also ions freely moving in the "free water" of the colloidal protoplasm and quantities circulating in the cell walls in addition. The total volume of this free space is difficult to calculate, but it must be assumed that ions have a more or less free passage through the protoplasm. This is also one of the conditions requisite for the appearance of a true Donnan potential (see below). But the conception of free space does not mean that the ions from the medium overflow into the protoplasm. The entrance through the plasma membrane is regulated by its sieve structure which favours smaller and less hydrated ions, and the passage through the tonoplast is probably still more restrictive (see below).

The ideas of free space comprise a further development of the author's and Robertson's (1, 11) conception of an initial nonmetabolic phase of salt accumulation. In investigations on the time course of salt accumulation (29) the author demonstrated that this initial nonmetabolic phase was developed faster than the subsequent active accumulation. This observation of a fairly high degree of mobility of ions in free space is in accord with observations of the easy flowing of sap through and from a tissue in which the respiration was inhibited by cyanide or fluoride. Needless to say, the filling of the free space from the medium is not an accumulation of ions in the free water of the protoplasm, but an eventual charge of the carrier groups. In higher concentrations outside, the nonmetabolic filling of the free space may mobilize quantities of salts which may be of some importance for the transpiration stream as shown by several investigators of the relation between salt uptake and transpiration (28, 64, 71, 73). It must, however, be kept in mind that the transpiration also indirectly supports the quantities of salts actively pumped into the roots (21).

Surface Potentials as a Guide to Exchange Reactions

Most recent investigators accept the Donnan principle for the calculation of the potential difference between the surface of the root and the surrounding medium, as introduced in earlier work by the author (9, 19, 20). Measurements of potential difference and its variation with pH and salt concentration were here regarded as a convenient means of studying the exchange processes between medium and plasma membrane. Vervelde (25) concedes the author's conception of "the root potential as being a direct consequence of an uneven distribution of ions across the root wall." Hope (23), too, accepts the idea of a mosaic structure of the surface with interspersed immobile ions. Similar ideas have been developed by investigators of the yeast cell (30, 31).

This ionic activity of the surface layer of the cell is apparently a fundamental property (11), regulating, not only the import and export of mobile

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ions, but also indirectly, by a sort of field influence, participating in the diffusion velocity, of noncharged molecules. The dissociated points of the membrane create, moreover, local pH areas around enzymes located in the surface, e.g., invertase, phosphorylases, etc., (30, 31). In special cases, e.g., the meristematic part of bean roots (23), diffusion of ions may be more pronounced and diffusion potentials will then participate in the development of potential difference .

In the absence of metallic cations in the surroundings of the carriers H*R* these will, if the pH is suitable, dissociate H ions. The root behaves then as an H ion electrode, because H*R* is far more concentrated than *R*OH. If a neutral salt M^+A^- is added to the medium, H^+ is partly exchanged for M^+. The negative potential of the root surface is consequently lowered. Each cation and each anion participating in the Donnan equilibrium will, of course, be distributed according to the finally attained potential (9). Tendeloo (32) has obviously misinterpreted the authors 'conception of the role of H ions in the Donnan equilibrium. Unfortunately, Hope & Robertson (24) have repeated this mistake.

At increasing H ion concentration of the medium the dissociation of H*R* is suppressed (and that of *R*OH increased), and it is possible, by following the decreasing potential difference, to calculate the apparent dissociation constant of the cation carriers H*R* (9). The author postulated that at potential difference = zero the dissociation of H*R* is approximately 100 per cent but just at the point of beginning suppression, from which a pK value of 1–2 was calculated. Robertson & Wilkins (see 24) and Vervelde (25) believe that dissociation of H*R* is completely suppressed at potential difference = zero and that the external H ions now invade the plasma membrane and thus eliminate the Donnan potential. According to the author's opinion, the overflowing of the membrane with the acid in the medium begins after the reversal of potential difference into positive values. The author is not convinced that his original calculations are not correct. They are also supported by the fact that acids of pK values amounting to 1–2, namely nucleic acids, are actually present in the root cells and in the surface of the epidermis (33).

Vervelde (25) erronously believes that the majority of the large molecules in the protoplasm are normally dissociated. Because of the low dissociation constants of most proteins a considerable fraction will be nondissociated at biological pH values. A higher degree of dissociation is confined to nucleotides, phosphatides, and a few basic components, as histones and ammonia bases. The simultaneous presence of all categories, of course, makes the titration curve of the protoplasm more extended than that of a single component. This may explain deviating results as to pH and potential difference. In the author's experimental material, the lower (*ca.* 40 mm. of 120 to 150 mm.) long roots of wheat seedlings, the results are consistent with the domination of strongly acid R^- compounds. The experimental results were satisfactorily explained from the principle of Donnan equilibrium and a factor a as an expression of the adsorption of cations in the protoplasm. This factor is specific

for each adsorbed ion. The reaction between ions and carriers was postulated to follow the law of mass action. If the observations are extended over longer time periods also, the production of organic acids in the tricarboxylic cycle participates in the nonmetabolic exchange of ions (see below). The extensive exchange of cations between the living root tissue and the medium, however, proceeds much more rapidly (equilibrium mostly attained in *ca.* 15 min.) (29) than a corresponding formation and consumption of organic acids, and is certainly primarily due to the exchange capacity of the structural components of the protoplasm.

Many examples of a selectivity, or of a specific absorption of cations, have been given in the past and in more recent literature (18, 34). Long ago it was shown in the author's laboratory that potassium is absorbed 24 times more rapidly than sodium by wheat roots. Potassium is absorbed 1.8 times faster than lithium, whereas potassium and rubidium, in spite of different atomic weights, behave very similarly (18). These and other results on selective absorption speak in favour of the existence of "specific carriers." Here, attention may be called to the fact that nucleic acids (which are shown to be present in the root protoplasm) (33), form nucleates with different cations having a specific solubility. As shown by the author, nucleic acids show strong geoelectric effects (35), a circumstance which regulates the electromotive power of the tissue and may be responsible for the geotropic response. Specific carriers, specific for single cations, e.g., sodium and potassium, or group specific, e.g., for potassium and rubidium, which are situated in the plasma membrane will, of course, largely control the selective absorption of the whole cell or tissue, even if the ions are transferred to other and less specific carriers in the interior. This is important as an argument against authors (6, 44) who believe that the cytochrome system, or any electron pump of ions, cannot promote a selective absorption (see below).

An experimental investigation of potential difference yields a fairly accurate conception of the frequency of ion carriers in the plasma membrane, but it may be expected that the concentration of carriers in the bulk of the protoplasm is of a similar magnitude, as actually shown by the calculations of Hope (23), Vervelde (25) and others. The path of the once absorbed ions is probably guided by "adsorption tracks," viz., a rapid interchange with other carrier ions, eventually arranged as ionized side groups along fibrillar molecules. Such an "adsorption gliding" of ions would highly promote the velocity of the process and thus be in accord with observed facts (see above).

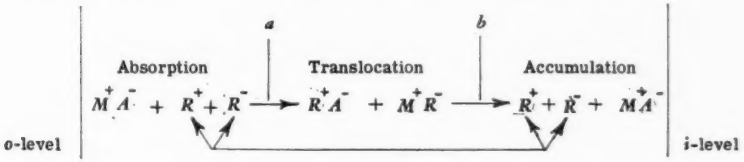
Hope (23) calculates the diffusion coefficient of KCl into the protoplasm of bean root meristem to 0.07×10^{-5} cm.^{2} sec.$^{-1}$. That the diffusion of ions through the plasma membrane is considerably retarded as compared with a free solution appears from the well-known fact that the membrane offers a high ohmic resistance. This fact, however, cannot support the idea of a "nonaqueous layer," once advanced by Osterhout and apparently still held, by him (17). A membrane which is perfectly permeable to water cannot, of course, be impermeable for inorganic ions, but owing to hydration and con-

sequently large overall diameter of many inorganic ions they will meet a comparatively high resistance to diffusion and electric transport. The pronounced contrast between the velocity of penetration of ions and of molecules has been repeatedly demonstrated, e.g., in the case of organic acids and fluoride (36), DNP (37), etc. All these substances were readily absorbed at low pH and suppressed dissociation.

The main points of entry of ions into the protoplasm are certainly the carrier groups R^- and R^+ and the free diffusion, indeed, is more a symbol of the free mobility demanded by the Donnan equilibrium. A number of authors, e.g., Rosenberg (7), Overstreet & Jacobson (3), and Hope & Robertson (24) now claim that ions enter the bulk of the protoplasm by exchange and diffusion. To what extent the transition of the plasma membrane is interwoven with "active transport" (see above) is difficult to say for the present. The delicacy of the plasma membrane as an instrument for active transport has recently been shown by various investigators [Rothstein and collaboratators (30, 31), Brown (38)].

Schemes of Active Absorption

The following Scheme A illustrates our concept of transport of ions from the surrounding medium (o-level) to the interior of the cell (i-level):



Scheme A

If reversible equilibria are assumed between ions and carriers (see above) the transference of a salt from the o-level to the i-level requires only little energy, as long as $(cMA_i/cMA_o) \leqq 1$. Observations on the blocking of the transport through the protoplasm by DNP [Arisz (15, 16), Butler (27)], however, show that metabolic processes may be involved in the maintenance of the structural tracks along which the translocation proceeds. Goldacre (39) believes that "protein molecules fold up at one end of the cell and unfold at the other and that this process results in osmotic work." This hypothesis is obviously a visualization of the carrier hypothesis, according to which the reaction ion+carrier→ion carrier is promoted at the o-level and the reversed reaction promoted at the i-level. Goldacre is, however, mistaken in claiming that the accumulation of ions is causally linked to the protein synthesis (see below).

For the performance of real osmotic work, either as active transport or as accumulation (see above), energy may be put in at a, at b, or in both. We have discussed active transport and accumulation as two largely synonymous

conceptions. A few words may also be said about secretion. This term commonly means an active exudation, whereas plain exudation is frequently used for passive out-going or leakage of solutes. Secretion is obviously a process of active transport or accumulation, too, but its use ought to be restricted to an accumulation of a solute on the outside of a cell. To speak of secretion of ions into the vacuole of a cell only leads to confusion. Salts are obviously accumulated in the cell sap. As to secretion and exudation it is important to bring home the fact that the concentration of a "passive" exudate from a cell is a function of its osmotic pressure, turgor extension, and relative concentration of the exuded substance in the cell. At low turgor extension (elasticity) a fairly concentrated solution may be passively exuded from a cell (26). Ignorance of the osmotic conditions has yielded much confusion in the discussion of sap movement.

According to the membrane equilibrium, viz., the principle of equal ion products on both sides of a membrane, one of the ions of a salt may be accumulated without extra expenditure of energy, but never both. Concommitantly, the accumulation of a free salt needs only active accumulation of one of its ions. Provided anions are actively accumulated the acid H*A* will spontaneously decompose the carrier complex *MR*, resulting in free salt *MA* and regenerated carrier H*R* capable of combining with new cations. The arrows in Scheme A indicate that the carriers R^+ and R^- may either circulate between the interphases or act as "adsorption tracks."

In growing cells the exchange capacity steadily increases and nutrient ions needed for synthetic work may thus be imported without the expenditure of extra energy. Bogen (10) characterizes this mechanism of import as "metaosmotical uptake." Metaosmotical absorption is regulated by the difference in free energy on both sides of a layer, e.g., a difference in the coefficient of solubility. Anions which are incorporated in elements of the protoplasmic structure, e.g., NO_3, SO_4, H_2PO_4, may be metaosmotically absorbed. Metallic cations may be imported in relation to the formation of organic acids, e.g., malic acid, which is abundantly accumulated in the cell sap of many cells. The formed acid reacts with the carrier complex *MR* thus releasing cations at *b* in Scheme A.

In all of these cases ions are continuously imported either owing to a continuous extension of the exchange capacity (formation of carriers) or a continuous production of organic acids. For true accumulation of neutral salts in the sap spaces of cells and tissues, special mechanisms are needed, however.

Mechanisms of Accumulation Other Than Anion Respiration

A number of possible mechanisms of true accumulation of neutral salts have been invented in model experiments, e.g., the machine built by Ernst & Homola (40) in which the maintaining of a temperature difference on both sides of a membrane results in the active transport of water from the warmer to the colder side, resulting in a rise of concentration in the former. However,

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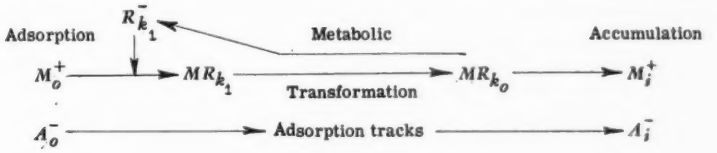
only those schemes which are based on the carrier principle are sufficiently anchored in known facts to be taken into earnest consideration. Besides "electronic salt pumps," akin to the anion respiration, only two schemes of possible mechanisms are, in the author's opinion, worthy of mention here, namely: (*a*) cyclical variations in the dissociation constants of the ion carriers *R* and (*b*) cyclical variations in the acid/base balance.

The total quantity of carried ions corresponds to the quantity of H^+ and OH^- furnished as exchange partners (as H^+R^- and R^+OH^-) and is consequently dependent on the dissociation constants of the carrier molecules. The dissociation of the surface layer of the protoplasm is partly regulated by the pH of the medium. More stationary differences in the state of dissociation, at unchanged constants, may exist if the pH of the medium is higher than the pH of the cell sap. The exchange capacity of the cation carriers will then be higher at the *o*-level than at the *i*-level (Scheme A), a circumstance favouring the import of cations. If the dissociation constant itself is changed from *a* to *b* (Scheme A) and back again, conditions are fullfilled for a real accumulation of both anions and cations, according to the following scheme ($k_1 > k_o$):



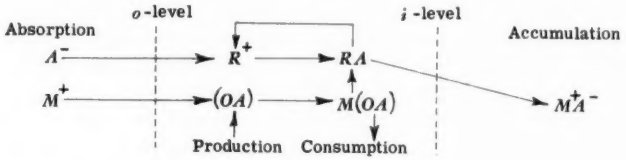
Scheme B

The scheme works similarly if the dissociation constants of the anion carriers are running through cyclical changes. If was earlier shown (11) that the binding of certain ions to the plasma membrane, e.g., Ca, Mn, BO_3, maintains a high electronegative potential difference, whereas potassium ions act in the reverse way. An actual increase of cHR in the plasma membrane, combined with a continuously running decomposition at the *i*-level, would do the same work. Real cyclic changes in the dissociation constants are known from the chain of glycolysis and respiration, e.g., in the phosphate metabolism where the adenosine phosphates have higher dissociation constants that inorganic phosphate and *k* of ATP^2 and ADP^2 are somewhat different, too. Goldacre's (39) hypothesis of cyclically folding and unfolding protein molecules would act akin to a cyclical variation of the dissociation constants.

Reversible variations in the acid/base balance have been studied by several investigators. Ulrich (41) and Burström (42) studied the mechanism of metabolic regulation of the internal pH of root tissue and found that an increased exudation of malic acid in the sap raised the import of metallic cations from the medium, primarily potassium. The process is promoted by high pH values of the medium. At low pH values organic acids disappear,

metabolized in the tricarboxylic cycle, and now the absorbed cations are again exuded (42). This reversible process of internal regulation of pH resulting from variations in the external pH proceeds (OA) fairly rapidly (43).

For the utilization of such a mechanism for the purpose of salt accumulation it may be envisaged that acids (OA) are produced near the *o*-level and again consumed at the *i*-level, according to the following scheme:

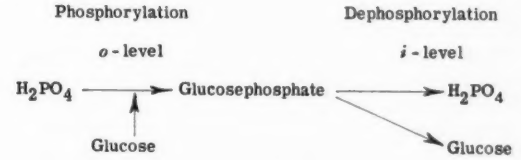


Scheme C

Some authors [Vervelde (25) Helder (44), Scott-Russell (45)] believe that this mechanism operates in tissues provided with a cytochrome system. This is, however, a mere speculation without sufficient experimental background. We know (see below) that the activity of the cytochrome system is intimately linked to the accumulation of ions in plant cells, but the actual physiological and biochemical evidence points to a more direct connection between the transference of electrons and the transport of anions. It must also be emphasized that organic acids produced in aerobic respiration may coenzymatically interfere with the cytochrome system [cf. Turner (46)]. Nevertheless, the Scheme C will be able to operate whenever the structural conditions are given. Such cases will have to be experimentally demonstrated, however.

Steward & Hoagland (47, 48, 49) once believed that both anions and cations are actively imported by aerobic respiration. Details about how such a mechanism would operate were not given. As mentioned above, it is a thermodynamical axiom that the energy of accumulation must be bestowed upon only one of the ions of a salt. Available experimental evidence supports the idea of anions being actively transported and cations passively dragged into the cell via carriers R^-. This mechanism is shown to work in roots (11, 50, 51, 52) and in storage tissue (1). As shown below, many cases of a seemingly active absorption of cations are explicable from different velocities of migration of anions and cations and from the simple fact there must be an electrochemical balance between both. If a mechanism of aerobic respiration actively moves anions it will, concomitantly, also move cations, and the real problem, which was put aside by Steward and Hoagland, but returned to its focal position by others is the disclosure of the site of the primary energy transference. The author has never denied the possible existence of special mechanisms of active transport of cations, e.g., according to Scheme C, but it has to be shown by conclusive experiments to what extent such mechanisms are actually operating simultaneously with, or as a substitute for the anion respiration.

A specific active absorption of anions can be expected in cases where an anion is caught by a reversible enzyme system in which anions serve as chemical components. It is often believed that phosphorylases are operating in the plasma membrane (30, 31, 53, 72). Monophosphate ions may be attacked by hexokinase and incorporated in hexosephosphate which, in the chain of glycolysis and subsequent metabolic transference, may be again released as inorganic phosphate somewhere in the interior of the cell. This mechanism (phosphorylation at the *o*-level, dephosphorylation at the *i*-level) would also be able to operate in the accumulation of glucose. Similar mechanisms may possibly operate on sulphate and nitrate, but it is still an open question to what extent such specific mechanisms are competing with or substituting the anion respiration.



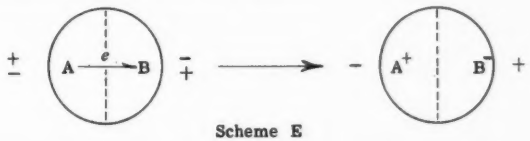
Scheme D

Definition and Properties of the Anion Respiration

The possible mechanisms of active transport quoted in the Schemes B, C, D, are based on the over-all Scheme A, implying cyclical variations of the capacity of ion carriers. The second main pathway of active transport is by means of structurally guided electrophoresis. Most widely known is anion respiration, which is claimed by Vervelde (25) "to be more advanced and embody more experimental data than any other theory."

Transference of electrons is the key to the chief redox systems in the living cell but biochemists have hitherto paid little attention to the electrochemical side of the problem. In the field of submicroscopical structure local potential differences created by electron transference are powerful agents of active transport of solutes as well as of locomotion, contraction and dilution of macromolecules, and micells.

The electrochemical effect of an electron transference between two molecules is illustrated by the following scheme.



Scheme E

The isoelectric field around the reactants A and B is turned into a bipolar field. The uniform swarm of ions surrounding the neutral system is now polarized. An anion is attracted to A^+ and a cation to B^-. This readjustment

of the external field compensates for the considerable electrostatic potential difference between A and B which would arise if no movable ions were present in the surroundings. The electrostatic potential difference would, in most cases, [as actually shown in the case of cytochrome oxidase (54)] raise an insurmountable barrier to the electron transference and the reaction would not take place. The presence of movable ions in the surroundings of a redox system, operating by means of electron transference, is consequently to be regarded as a coenzymatic effect.

This coenzymatic effect of movable ions around a system $A \overset{e}{\rightharpoonup} B$ can now, under appropriate structural conditions, be turned into an effective ion pump. The term "anion respiration" was created in the early thirties. It was experimentally shown that in roots of wheat seedlings the absorption of anions is quantitatively related to one fraction of the aerobic respiration. A second fraction of the respiration, the ground respiration, was not directly related to the transport of ions. It was shown at an early date that anion respiration is conducted by enzymes exhibiting all the features of active iron (51). Observations on the extreme sensitivity to cyanide (36, 51), the light-reversible sensitivity to carbon monoxide (60), and, finally, the spectroscopically observed co-variation of anion absorption and oxidation-reduction level of the cytochrome system (43, 57, 59), definitely proved the identity of anion respiration with the activity of the latter. Peroxidase, which is also present in roots, does not participate in anion respiration (43).

According to Scheme E, an anion is attracted by B when it is oxidized, viz., loses one electron. Provided a barrier exists between A and B (as indicated by the dotted line) which slows down the free diffusion inside of the system, anions will be moved from right to left, if electrons are intermittently moved from left to right. In the complete cytochrome system electrons are moved successively from succinodehydrogenase to cytochrome-*b*, to cytochrome-*c*, to cytochrome *a* (cytochrome oxidase), and then to the final acceptor, molecular oxygen. The three main cytochromes, discovered by Keilin in 1925, are probably aided by other factors. Keilin assumed a modification of cytochrome-*a*, Slater found evidence for a factor cooperating with *c* and *b*, and the author described a new cytochrome-*dh*, present in roots and related to *b*. The complete system acts as an electron ladder leading from the dehydrogenase to oxygen. From an electrochemical point of view the cytochrome system behaves as an electric battery with its negative pole at the site of the oxidase, its positive pole at the place of cytochrome-*b*, as shown in the following Scheme F. One anion is attracted by the cytochrome oxidase each time it emits one electron to a molecule of oxygen adsorbed on the surface of the enzyme (43). The anion may be considered to be advanced to the next cytochrome in the ladder, when, by means of thermal rotation, it receives a new electron from the latter, and so on, until the anion is forwarded to cytochrome-*b*. Here is the journey's end because there is no further structural path to guide the transport of the anion. It is released from cytochrome-*b* at the moment when one hydrogen atom, detached from succino-

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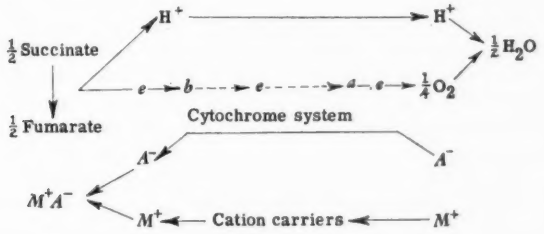
dehydrogenase, is half oxidized to a proton. The released anion may immediately combine with the proton, but the formed acid probably reacts rapidly with a cation carrier MR (see above) in the surroundings, thus forming the neutral salt M^+A^-. The proton finds its way in the reverse direction to the anion along the "cytochrome battery" and joins the activated oxygen, forming water. The complete circle of electrophoresis is hereby closed, as a consequence of which no metallic cations are lost from the cell under normal working conditions (12).



Scheme F

It was postulated that the shuffling of anions along an electron ladder needs a cooperating diffusion barrier, placed somewhere on the track, to prevent the back-flow of once accumulated anions. The structural character of this barrier is not yet known, but experiments with certain inhibitors have shown that biochemically it is closely related to cytochrome-*b*, thus, near the bottom of the electron ladder. Inactivation of cytochrome-b, viz., an interruption of the electron transfer from the dehydrogenase to *b*, completely stops the ion accumulation. Such inhibitors are DNP and fluoride. As these inhibitors do not affect the further electron transfer to *c* and *a* and from here to oxygen, these cytochromes are obviously not able to carry the anions safely behind the barrier. But *b* alone is unable to accomplish such an accumulation. This appears from the fact that cyanide inhibits only cytochrome oxidase, but leaves cytochromes-*c*, *b*, and *dh* largely intact and able to carry on a reduced oxidation. The anions must consequently be caught by cytochrome oxidase and released by cytochrome-*b* to be really accumulated. Only the intact cytochrome system is thus capable of active salt transport.

The oxidation of cytochrome-*b* is reversibly coupled to formation (or decomposition) of ATP (57). This is the reason for its sensitivity to DNP. It has been suggested (43, 59, 61) that the key position of cytochrome-*b* has some effect upon the function of ATP as a "protector" of normal protoplasmic structure. The author suggests still another scheme, according to which high energy phosphate supervises the formation of special carriers which take over the anions released from cytochrome-*b* and carry them to the place of accumulation.

Discovering the key position of cytochrome-*b* and its linkage to the high

energy phosphate metabolism excludes objections raised by Robertson (1), Bartley & Davies (74), Overstreet & Jacobson (3), and others who suggest the need for an additional mechanism to explain the effect of DNP. The critical or even polemical attitude against the theory of anion respiration once held by Steward and Hoagland had overshot the mark, but its tenacious survival in modern text-books (62, 63) unfortunately bears evidence of the superficial treatment to which these difficult problems are often exposed. A number of erroneous interpretations have been answered in the foregoing. In error is the pertinacious belief that accumulation of ions is causally linked to growth. It was shown above that a growing protoplasm metaosmotically imports most of its demands for nutrient ions. That real accumulation in the cell sap of outgrown cells, e.g., cortex cells 50 to 100 mm. from the short tip zone of growth, proceeds independently of growth is an easily recognizable fact. It has, furthermore, been convincingly shown (36) that extension growth may be inhibited even by auxin (in roots) without disturbance of the accumulation mechanism. The observed fact that slices of storage tissue start growing simultaneously with accumulation of salts, (and this is one of the arguments advanced by Steward) must not necessarily mean that growth is the promotor of accumulation. The causality may well be the reverse, viz., growth stimulated by the presence of salts. If the activity of the cytochrome system is one of the fundamental conditions of growth, and this can easily be shown by using cyanide inhibition (36), all factors promoting the activity of this system will also indirectly promote growth. We know at present that salts are indispensable coenzymes for the activity of the cytochrome system. It has been clearly shown that the oxidation-reduction level of the total system is regulated by added salts (57, 58, 59). Our conclusion is, consequently, that observed linkages between salt accumulation and growth depend upon the activation of the cytochromes by the salt, followed by parallel growth and salt accumulation. Growth may be uncoupled from anion respiration because growth is also dependent upon a number of other processes. This uncoupling affects the accumulation of salts only as far as the activity of the cytochromes is affected.

Quantitative Relations Between Anion Respiration and Salt Transport

The existence of quantitative relations between the absorption of anions and the cyanide-sensitive fraction of aerobic respiration has been shown in a large number of investigations from different laboratories (6, 7, 50, 51). Inability to observe quantitative relations may depend on low concentration of cytochrome and difficulties in measuring small differences in oxygen consumption. The author has recently shown (43) that the intensity of anion respiration varies linearly with the concentration of cytochrome-*b* and that the development of the cytochrome system in roots of cereals is controlled by a number of factors, e.g., the supply of mineral nutrients, climatic conditions, etc. One cannot speak of anion respiration in an object before its

cytochrome system is investigated, at least by determination of the cyanide-sensitive fraction of the respiration. The pre-treatment of the objects is, of course important e.g., a low level of initial salt content, attained by continued soaking in distilled water. Glucose, of course, serves as the fuse (21, 73).

One of the most obvious pit-falls is the fact that the intensity of anion respiration is dependent not only on the quantities of salts absorbed from the medium but also on the quantities of movable anions circulating inside the epidermis, viz., in the free space (see above). The author, four years ago, devoted a special investigation to the elucidation of this fundamental problem, but nevertheless some recent investigators believe that "anion respiration operates only if salts are present in the medium" [van Andel (64)].

Robertson called attention to the fundamental fact that, according to the over-all reaction $4H^+ + 4e + O_2 = 2H_2O$, four anions corresponding to four electrons (see Scheme F) should be transported for each molecule of oxygen consumed. As a matter of fact, the quotient (M anions/M O_2) or Q an/O_2 (36) can rise to about 4, but higher values have hitherto not been observed. Robertson attained the maximum value in high salt concentrations. Normal nutrient solutions render values down to about 1 or still lower. The highest value obtained by the author for wheat roots is about 2.5.

Robertson made no attempt to explain why anion respiration is seemingly less effective if dilute solutions are offered. The energy relations are indeed highly profitable for the attainment of very steep gradients, because even a 100-fold accumulation consumes only about 2 to 3 per cent of the free energy of the system. The explanation of the low Q an/O_2 values was given by the author's investigations of the "distilled water respiration" (d.w.r.).[2]

It was shown (36) that roots directly transferred from a nutrient solution to distilled water do not immediately slow down their anion respiration to zero, but show a certain "idling." This idling is, however, not caused by a re-accumulation of escaped salts, as was originally believed (52), because the passive leakage of anions from the root surface is insignificant. A continued observation of roots transferred from a nutrient solution to distilled water revealed a slowly sinking idling of the anion respiration. An almost complete inhibition of d.w.r. was attained after treatment with dilute solutions of minerals ac *ca.* pH 3.3. to 3.5). Transference to slightly alkaline solutions (potassium bicarbonate) considerably increased d.w.r. Chemical analysis of the expressed sap showed a rapid decrease of the percentage of organic acids (primarily malate) in an acid medium but a considerable rise in an alkaline medium (43).

The investigations convey the conclusion that d.w.r. reflects the coenzymatic effect of anions circulating in the tissue. These "native anions" are partly previously-absorbed salts remaining in the free space, partly anions of organic acids produced in the protoplasm. As shown above it is possible to distinguish between these two categories and to calculate their percentual participation in the total anion respiration. At a normal import of nutrient

ions, and an external pH of *ca.* 6, the anions of organic acids absorbed 50 per cent (at a maximum) of the total capacity of the cytochrome system (36). The circulating ions in the free space (see above) impound about 25 per cent. Only about 25 per cent of the total capacity of the cytochrome system is then available for active import from the dilute medium, corresponding to Q an/O_2=*ca.* 1. Higher concentrations in the medium augment this fraction.

This experimental analysis of d.w.r. satisfies all of the observed facts. It is still postulated that four anions of mineral salts or organic acids are accumulated, primarily in the cell sap (see below), for each molecule of oxygen consumed. It is further postulated that the cytochrome system is present in all, or in most, cells of the tissue (root tissue, storage tissue, and other accumulating tissues). In the epidermis, salt ions are directly accessible from the medium. In the interior, ions are accessible from the free space, viz., cell walls and the "free water" of the protoplasm. At the pH values normally prevailing in the protoplasm (in wheat roots *ca.* 6.2) the organic acids produced in the respiratory cycle are completely dissociated. Because the electrochemical transport mechanism of the cytochrome system transports anions of any kind, a competition goes on between organic and inorganic anions which is regulated by the relative concentrations and by the mobility in an electric field. The latter circumstance makes the competition rather selective (see above) and, as a rule, favours small inorganic ions in comparison to larger organic anions. This fact explains why comparatively large quantities of neutral salts are accumulated in spite of the low initial concentration in the medium, normally remaining at *ca.* 0.001 *M*, as compared with the probably considerably higher availability of anions of organic acids. The theory of anion respiration actualizes the problem of active accumulation and transport of organic acids in plant tissues. This problem awaits future investigation.

The leading idea of our discussion, viz., anion respiration proceeding in the bulk of the tissue and not only in the surface layer, explains why the theoretical value Q an/O_2=4 is attained only at such high concentrations of salt anions in the medium that the competitive effect of "native anions" is practically ruled out. In this case the salt anions completely dominate the cytochrome system of the epidermis and, because they also invade the free space inside of the epidermis, almost completely dominate the anion respiration of the internal tissue, also. At lower salt concentration in the medium the native anions share an increasing percentage of the total transport power, as a consequence of which Q an/O_2 is lowered, because "anion" here means only the anions absorbed from the medium. These facts are unfortunately ignored in most recent investigations on active salt accumulation, a circumstance inspiring many erroneous interpretations and even the denial of the existence of a salt respiration [cf. (12)].

Our discussion of d.w.r. also explains the observed differences of Q an/O_2 with different anions, e.g., NO_3, Cl, SO_4 (50). A slower mobility of one anion will act in the same way as dilution, viz., it will lower the competition with

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native anions. The presence of slow movable cations correspondingly reduces Q an/O_2 because the total process of salt accumulation requires a continuous import of cations also, unless the carriers taking over anions at cytochrome-*b* (see above) will not be blocked by nonreleased anions. In all these cases of aggravated transport of ions the native anions will share a larger part of the assembly, and Q an/O_2 goes down. A successful demonstration of the constancy of Q an/O_2 is subsequently subjected to conditions in which the quantity of native anions does not vary appreciably in the scope of the experiment. This is a further warning against too rash conclusions arrived at from unsuitably planned experiments.

The Cytochrome System in Roots

The author has recently demonstrated in great detail the simultaneous variation of the oxidation-reduction state of the cytochrome system and the salt absorption (54 to 59). It was earlier shown that the constancy of Q an/O_2 is maintained if the capacity of the cytochrome system is gradually diminished under the influence of cyanide (36). These experiments were corroborated by new experiments showing a quantitative parallelism between the percentage of cytochrome-*b* and the ability of salt transport (43). Other recent experiments show a complete parallelism in the time course of (*a*) salt absorption, (*b*) displacement of the oxidation-reduction balance of the cytochrome system, and (*c*) the inward diffusion of oxygen, as reflected in the velocity of oxidation if roots are transferred from complete anaerobiosis to an aerated solution.

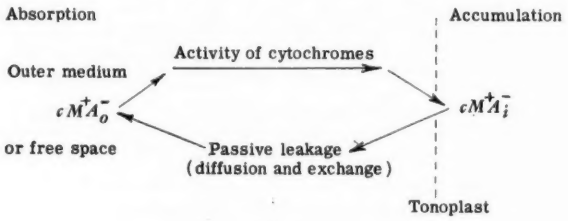
The theory of anion respiration is based on the experimentally shown coenzymatic activity of anions on the cytochrome system. Without movable anions there is no cytochrome respiration. This coenzymatic effect is probably universal, presumably present also in other redox systems, acting by means of electron transference. But this universally stimulating effect of salts must not necessarily be combined with salt accumulation. Most animal cells show no obvious salt accumulation. Salts may be simply loosely attracted by the common activity of the electronic systems. Only plant cells are apparently provided with mechanisms turning the universally-occurring shuffling of anions against electrons into a polar stream, viz., into osmotic work. According to Gorham & Clendenning (65) anions exert a coenzymatic activity also in chloroplasts. The presence of cytochromes [*f* and *b*, according to Hill (57, 66)] is of considerable interest in this respect.

The experiments on anion respiration clearly show that in roots and storage tissue the coenzymatic effect of organic anions produced in the mechanism itself is not sufficient for a maximum activity. For this free salt anions are required. By analogy, it may be suspected that salts are necessary requisites in very active animal cells, also, e.g., in the heart muscle. This is a physiological-biochemical problem of wide scope which is scarcely touched upon in the literature yet. We know that the activity of cytochrome-*b* is indispensable for salt accumulation. We know that salts are accumulated in

the large sap space of the cells. We know that the ascending sap in the root xylem, appearing as bleeding from decapitated young plants, is frequently but not regularly, more concentrated than the external solution. We do not know the actual concentration of salts in the protoplasm itself, but it is sometimes assumed that it is lower than in the cell sap (13). This is not so important, however, because we know that the large quantities of salts which are accumulated in the sap space must pass through the protoplasm and be exuded through the tonoplast. We know finally that the property of salt accumulation is not confined to growing cells and that the central mechanism of accumulation is not directly correlated to protein synthesis. Cytochromes are, on the other hand, doubtlessly formed as a result of the total building up of the protoplasm. We do not know how long a complete cytochrome system persists in ageing cells and if its presence is an indispensable condition of the retaining of salts. It may be that in older, but still turgescent cells, the tonoplast loses most of its exchange permeability for salts. These questions will have to be investigated.

In the fully developed but still comparatively young cells of the root cortex the tonoplast is certainly reversely permeable to salts. If well nourished root systems are held for several days in distilled water the stored quantities of salts, e.g., potassium nitrate (21), rapidly disappear, as does also the stored sugar. This emptying of the sap spaces has been observed by several investigators. It may also be followed by observing the lowering osmotic pressure (21). If the starved roots are replaced in a nutrient solution the sap spaces are again filled, and the osmotic pressure rises.

These observations of the reversibility of the salt accumulation can only be explained as a steady-state, viz., a balance between an active accumulation and a passive leakage, according to the following Scheme G



Scheme G

Provided that the uphill and downhill processes are constantly at work, an approximate balance SS = (c. $M^+A^-{}_i$/c. $M^+A^-{}_o$) will be maintained. Under constant working conditions, a decrease of c. $M^+A^-{}_o$, viz., the salt activity in the free space, will promote an increased passive leakage, a situation observed in starving tissues. An increase of c. $M^+A^-{}_o$ will concommitantly promote the active accumulation. The experimental evidence, e.g., the exudation through the vascular epithelium (see below), supports

the conclusion that the actual salt concentration in the free space is higher than the concentration of a normal nutrient solution. It may be assumed that the anion respiration already present in the epidermis lifts the concentration in the free space to a level corresponding to the approximate steady-state balance SS.

We now face the problem of the transference of the anions from the protoplasm in which the cytochrome system is working, into the cell sap. This problem is intimately connected with the important question as to the localization of the cytochromes in the cell.

The Possible Localization of the Cytochromes in the Cell

Studies in the kinetics of the cytochrome system (55) show that there is a very rapid through transference of electrons from *b* to *a* to oxygen. There is, on the other hand, experimental evidence supporting the idea that the system is operating in two sections, $a-c$, and $c-dh$ ($-$dehydrogenase), respectively (43). These sections normally intimately cooperate, but may also, under the influence of inhibitors (see above), be uncoupled from one another. The Slater factor is believed to serve as a sort of collector of electrons. These facts are mentioned here because the possibility cannot be theoretically excluded that the high potential section ($a-c$) is located separately from the low potential ($b-dh-$dehydrogenase) section, provided one or both of the sections are movable and can rapidly exchange electrons and anions. The accumulating evidence regarding mitochondria are, however, more in line with the assumption that the total system is a rather compact unit forming an integral part of the protoplasmic structure.

The author demonstrated that living roots oxidize reduced cytochrome-*c* in the medium (54), and believed this to be evidence pointing to a localization of the cytochrome oxidase in the plasma membrane. It was pointed out that a cytochrome system serving as a constituent of the plasma membrane would, in a simple way, explain the barrier against back-flow which is a necessary complement to the coenzymatic activity of the anions (see above). In the meantime, accumulating evidence (53, 74) points to mitochondria as the site of the cytochrome system even in plant cells. These observations, however, refer to homogenized cells in which, according to Perner & Pfefferkorn (67), the mitochondria regularly are irreversibly disintegrated, and in which artefacts are formed whose reaction is somewhat similar to mitochondria. If the cytochrome system is surface active it will have the tendency to move into surfaces, e.g., into the layer coating granular artefacts of the homogenized protoplasm. Only an incisive investigation of the whole complex of problems can make the final decision as to the localization of the cytochrome system in the living cell. A number of enzymes have been attributed to the surface layer, e.g., invertase, hexokinase, peroxidase, ascorbic acid oxidase, etc. [see (53)]. What has partly upset the author's previous belief in the cytochrome system as an integral part of the outer plasma membrane is not so much the recent communications on mitochondria, but more the de-

velopment of the conception "free space" (see above) and of the steady-state balance between salts in the free space and in the sap space (vacuole). This theory is incompatible with the idea of the bulk of the protoplasm as the site of heavy salt accumulation. It is more likely to assume that the main process of accumulation is enacted near the boundary between protoplasm and vacuole, an assumption repeatedly advocated by Arisz (13 to 16). Owing to the thinness of the plasma layer and the rapid transport through it (see above) this does not necessarily mean a fixed structural attachment of the cytochrome system. The accumulation of anions from the protoplasm into the vacuole is in accord with the common acidity of the sap and the secondary release of cations from the protoplasm. The actual reversibility of this process (see above) argues against a direct release of anions from cytochrome-*b* into the vacuole, viz., against the tonoplast as the site of the cytochrome system. We are again returning to the carrier principle. If cytochrome-*b* surrenders the anions to carriers, which are specialized factors in exchange reactions in the tonoplast, this would explain both the accumulation in the cell sap and the passive leaking out according to our Scheme G.

From this discussion of the problem of localization of the cytochrome system in the cell it appears that there is now no real discrepancy between the author's theory and Arisz' conception of a "secretion" into the cell sap. Arisz accepts the anion respiration as an instrument of accumulation. He has pointed to the existence of a phase of transport of salts through the protoplasm which the author, supported by the recent development of the idea of "free space," has identified with conditioned diffusion and movement along exchange tracks. Arisz' secretion into the vacuole, finally, is identified by the author with the last stage of accumulation, linked to cytochrome-*b* and most probably including a carrier system adapted for transference through the tonoplast. The reader will note that the question as to the precise localization of the cytochromes is left open for the present. It is even possible that the cytochrome unit acts as a movable carrier between the outer and inner plasma membranes. But there are certainly other carriers R^+, too.

The Sap Flow Through the Root Tissue and into the Xylem

The author showed a few years ago (26) that salts, which are once absorbed through the epidermis, are comparatively freely flowing through the tissue and that this movement is not dependent upon respiratory or glycolytic processes. Together with the earlier conception of a nonmetabolic start phase of salt accumulation and the recent ideas of a "free space" (see above), these observations constitute a solid basis of our knowledge of the sap flow into the xylem. According to our Scheme G the cytochrome system of each cell of the root tissue not only pumps salts into the vacuole but also, via the reversible balance visualized in Scheme G, lifts the salts circulating in the free space to a level normally exceeding that of the surrounding medium. This circulating solution flows out through the "vascular epithelium" (26) and appears as bleeding from decapitated plants. Lack of space impedes a

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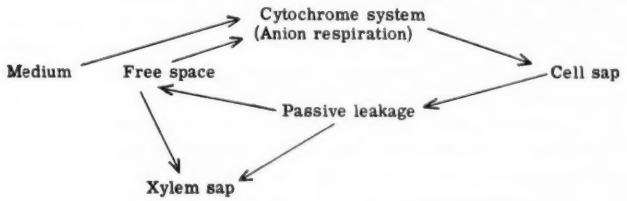
further analysis of the osmotic relations of the process. The fact that the sap flows out in the direction of the central vessels and is not returned to the medium has been explained from the obviously low activity of anion carriers in the surface layer of the epidermis (26), combined with a low salt permeability. A more pronounced equivalency between R^- and R^+ and a higher common salt permeability of the plasma membranes of the vascular epithelium would, in a simple way, explain the observed facts. Special investigations are needed here.

Van Andel (64) claims that "salts are given off from the whole root tissue" and is in agreement with Hylmö (28) who emphasizes the movement of salts in the free space as a source of ascending sap. These investigators, however, overlook the consideration that salts moving in the free space have actually attained a certain level of accumulation, according to Scheme G. If we postulate such an intermediate level of accumulation of the salts in the free space the discrepancy between the author and Arisz as to an "active secretion" into the vessels (5, 13 to 16) disappears. The only thing we actually know is the fact that the exuded bleeding sap can be considerably more concentrated than the external medium. But this is not, against the views of Arisz (15), van Andel (64), and others, a conclusive proof of active secretion (see above). Hylmö (28) believes that salts are actively accumulated in the still living lower parts of the vessels, the xylem sap thus corresponding to the cell sap of closed cortex cells. This hypothesis does not consider the fact that the sap exudation is not stopped by inhibitors of the anion respiration.

Broyer (68) showed that anions, which are absorbed from the medium, may migrate through the protoplasm to the xylem without an intermediary accumulation in the vacuoles. Arisz (15) came to similar conclusions as to the migration of salts in the leaves of *Vallisneria*. Earlier, Weevers (69) came to similar conclusions as to the transport of sugar. In agreement with the author's results on the bleeding of wheat seedlings, Arisz, too, finds no inhibition of the transport by KCN or DNP. But a nonmetabolic flow of salts through the free space of the cells cannot be explained otherwise than as a movement, by means of diffusion, ion exchange (eventually along adsorption tracks), and streaming in direction of the concentration gradient. In order to get a continuous flow a certain level of concentration must be maintained, e.g., by leaking out from the vacuoles, as actually observed during starvation. This slow, passive emptying of the total salt stores explains the slow cessation of bleeding under anaerobic conditions or under the influence of cyanide, etc. Only if anion respiration is intact and pumps in new quantities of salts can the out-flow through the vessels proceed. Even in the absence of salts in the medium anion respiration speeds up the internal transport because it, according to Scheme G, acts as an efficient mixer of the free space. The author is still unable to find any conclusive arguments in favour of an active "tissue secretion," as claimed by Arisz (15, 16), van Andel (64), and others because he cannot envisage an active process which is insensitive to anaerobiosis, cyanide, and fluoride, and which has a considerably lower temperature

quotient than metabolic processes (26). The xylem sap is apparently a non-metabolic "canalized leakage" from the vascular epithelium (26), but the total free space is involved. Active secretion and active transport can be simulated by a previously working anion respiration, which raises the concentration level of salts, not only in the large storage chambers of the vacuoles, but also to a certain degree in the free space, viz., in the inter-intracellular solution of salts circulating inside the epidermis barrier.

The conclusions arrived at in this section may be summarized in the following Scheme H. For each molecule of oxygen consumed the cytochrome system accumulates four anions. The process may proceed in any cell and the anions may be absorbed directly from the medium (in the epidermis), from



Scheme H

the free space (which is charged indirectly from the medium or from escaping cell sap), or from the organic acid metabolism linked to aerobic respiration. According to the steady-state prevailing between cell sap and free space (see above and Scheme G) the latter may, in the interior of the tissue, acquire a higher level of concentration than the medium. The xylem sap is regarded as exuded contents of the free space, and may secondarily be fed from the contents of the vacuoles.

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REDISTRIBUTION OF MINERAL ELEMENTS DURING DEVELOPMENT[1]

By R. F. Williams

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia

In the course of a recent address on the control of growth and reproduction in plants, Gregory (13) drew attention to the fact that over 90 per cent of the nitrogen and phosphorus taken up by the developing cereal plant had been accumulated when the dry weight was only 25 per cent of the final value. This store of accumulated nutrient was the reserve on which all later growth and development depended, and its level determined the final yield. The nitrogen and phosphorus which was set free from the senescent leaves and tillers was reutilized for production of further leaves which, in turn, provided for the development of the inflorescence. Gregory also stressed the significance of the meristems with their potentially unlimited demand for nutrients, and their capacity to initiate "internal starvation" and senescence. These observations serve very well to introduce the review which is to follow for, in examining the evidence for the redistribution of mineral elements during development, the reviewer has attempted to relate this to the wider problems of growth.

From the outset it should be emphasized that we are primarily concerned with the demonstration and explanation of net losses of mineral elements from specific plant parts. To be relevant, therefore, papers for review had to cover two or more plant parts at several stages of growth. Relatively few of the many papers on the mineral composition of plants fulfil these requirements.

It seems probable that some elements simultaneously enter and leave the same organ in the course of the normal metabolic flux, and may be regarded as being redistributed from organs which are still undergoing net intake. Such phenomena are not considered here, and it is for this reason that little reference is made to recent work with radioactive tracers. A borderline case is provided by Phillis & Mason (34) who found diurnal variations in the mineral content of the leaf of the cotton plant. The six elements studied showed increases by day and no change or decrease by night. Although the existence of dew losses complicated the picture, it does seem likely that phloem export was responsible for some losses even before maximum absolute contents had been attained. This evidence is a reminder that there can be no hard and fast line between redistribution resulting from normal metabolic activity within the organ considered and redistribution which is conditioned by senescent changes and by demands which arise externally to that organ.

[1] The survey of the literature pertaining to this review was concluded in August, 1954.

Potential rates of translocation whether via the xylem or the phloem would seem to be very much greater than required by redistribution in this last sense, so that the mechanism of transport also falls outside the scope of this review.

Classical work on the utilization of seed-reserve nitrogen was reviewed by Chibnall (8) in the early chapters of his book, and Miller (24) covers early work on the migration of mineral elements from the leaves of deciduous plants prior to leaf fall. This indicates that nitrogen, phosphorus, potassium, magnesium, and iron may migrate in this way, sometimes to the extent of 90 per cent of the maximum amount present. Olsen (26), with beech leaves, recently obtained evidence of net export of nitrogen, phosphorus, and potassium, but not of magnesium. Calcium, manganese, iron, and silica increased steadily throughout the life of the leaf. Miller also cites examples of migration from stem to ear in gramineous plants. Knowles & Watkin (16) made an early and very thorough study of nutrient intake and distribution in the tillers of wheat data being given for nine occasions commencing seven weeks before ear emergence. Their data indicate marked transport of nitrogen and phosphorus and some transport of potassium and calcium from straw to ear. The Trinidad studies of transport in the cotton plant build up a general picture of concentration gradients between "source" and "sink." That redistribution of nutrients is quite a general phenomenon is taken almost as axiomatic, and some new examples of redistribution were established. Mason & Maskell (20) concluded that nitrogen, phosphorus, and most ash constituents are redistributed to the boll via the phloem, but that calcium is not mobile. They review some of the earlier literature on these points. They also established the redistribution of nitrogen from storage in the bark and of phosphorus from the bark and wood (21). Phillis & Mason (33) found that, following anthesis, several elements were transported from the corolla into the fruiting branch, and they quote the earlier and parallel evidence of Schumacher (39) that as much as two-thirds of the nitrogen present may be exported from the corolla within a period of 24 hr.

In view of the wide range of physiological age to be found among the cells of some organs such as roots, it is not surprising that redistribution should occur between cells and tissues within an organ. Thus, Reid (37) has shown that cells only 15 mm. from the root tips of cowpea seedlings had already lost 5 per cent of their nitrogen and 14 per cent of their phosphorus. This observation needs confirmation, though the nitrogen loss occurred in all five tests, and the phosphorus loss in four out of five.

Redistribution in ontogenesis.—The most extensive body of data providing information relating to the redistribution of mineral elements during development is that which came from the laboratory of the late Dr. A. H. K. Petrie at Adelaide, South Australia. Since that work no longer continues and Petrie's colleagues are now scattered, it is appropriate that the contribution of this group should be at least partially reviewed within the bounds of a

single article. The objective of the work was the study of certain of the progressive physiological changes which occur in plants during ontogenesis. Petrie's approach was to alter the ontogenetic drifts by experimental means, and in the earlier work with gramineous plants this was done by exploiting the fact that ontogeny is affected by the nutrient supply. The effects of varying the initial supply of nitrogen were studied by Ballard & Petrie (3) and Petrie (30), and similar studies with phosphorus were made by Williams (52, 53, 54). Some insight was thus gained into the way in which the general pattern of growth can be modified by certain factors of the external environment. In later studies with the tobacco plant, Petrie achieved alteration in ontogeny by preventing inflorescence development. Changes in dry weight and leaf area were reported by Petrie, Watson & Ward (32), and changes in nitrogen content by Watson & Petrie (49). In all of this work the significance of relationships at higher levels of organisation of the plant body was stressed. Such relationships include those among different organs of the plant and, in particular, it was apparent that the development of the inflorescence profoundly influences the later ontogeny of other organs such as the leaves. Studies of the flax plant made by Tiver (43) and Tiver & Williams (44) contributed something to the general picture, though they are rather less relevant to the subject of this review. In their case the experimental variable was water supply, as was also the case in a further study of tobacco published after Petrie's death [Petrie & Arthur (31)].

Nitrogen and phosphorus.—Evidence on the redistribution of nutrients given by Petrie (30) is limited to absolute and relative amounts of protein and nonprotein nitrogen and of total phosphorus in the leaves of wheat and Sudan grass. He showed that the absolute amounts of these constituents were increased with increasing nitrogen supply, N_1, N_2, etc., and that the attainment of maximum content was delayed for the nitrogen constituents. Net export of nitrogen and phosphorus from the leaves of wheat was very great as may be seen from the following figures.

	Treatment			
	N_1	N_2	N_3	N_4
Per cent export of N	84	86	86	82
Per cent export of P	85	91	90	89

These are percentages of the maximum values, and they show a surprisingly small dependence on the level of nitrogen supply. Parallel figures for the leaves of Sudan grass are:

	Treatment		
	N_1	N_2	N_3
Per cent export of N	67	65	43
Per cent export of P	74	76	50

In this case net export was less drastic and it is reduced with the highest level of supply. Data for the third leaf of the primary shoot of wheat give a picture which is similar but condensed in time. However, there is a suggestion that net export of nitrogen may occur slightly earlier with N_2 than with N_1.

In seeking to interpret these results, Petrie had at his disposal the dry weight data already presented by Ballard & Petrie (3). These include values not only for leaves, but also for stems, roots, and inflorescences. These workers recognized three phases in the life-history of a single vegetative organ: (*a*) an initial period of growth which they termed adolescence; (*b*) a period of constant weight after the cessation of growth, i.e., maturity; and (*c*) a period of decline in weight and of internal disorganization termed senescence. A knowledge of the redistribution of mineral elements within the plant is clearly relevant to an understanding of the factors which determine the onset of senescence. Petrie (30) discussed the determination of the amounts of nitrogen and phosphorus in the leaves under seven headings: (*a*) the direct effect of the external concentration of a nutrient on its rate of intake by the whole plant; (*b*) the rate of growth of the leaves as a determinant of their capacity for intake; (*c*) the rate of growth of the other parts of the plant and the nature of their development as these affect the net export of nutrients from the leaves; (*d*) the capacity of the root system to take in nutrients at a rate sufficient to meet all demands; (*e*) the possible effect on protein synthesis of a declining rate of carbon assimilation; (*f*) the possibility of other internal factors leading to a decreased rate of protein synthesis or an increased rate of hydrolysis; and (*g*) the effects of starvation with respect to some nutrient other than nitrogen. With respect to (*c*), Petrie pointed out that the later development of the stems and inflorescences would cause the rate of export to increase with time until senescence sets in. He also suggested that the lesser rate and extent of the export of nitrogen from the leaves of Sudan grass than of wheat was due to the relatively small inflorescence weight ratio in Sudan grass.

Williams (52) studied the effects of varying phosphorus supply on the growth of the oat plant and its parts. His conditions were such, however, that plants receiving his higher levels of phosphorus, P_2 and P_3 quickly exhausted their nitrogen supply, and this hastened flowering and senescence generally for these treatments. By contrast, Sommer (40) has shown that limited supplies of both nitrogen and phosphorus will hasten senescence when other nutrients are in adequate supply. As was to be expected, Williams (53) found that the net export of nitrogen from the leaves was delayed by phosphorus deficiency, and, for his two experiments, the percentage export figures for nitrogen are:

	Treatment		
	P_1	P_2	P_3
Experiment 1	80	78	78
Experiment 2	65	78	73

Third leaf data for Experiment 1 show maximum nitrogen contents fairly early, and 93 per cent of this was lost to other parts of the plant. In considering the factors governing the amounts of nitrogen in the leaves, Williams retained the first four headings of Petrie's analysis (see above), but grouped the other three under one heading, (*e*) internal changes in the leaves leading to a decrease in the net rate of protein synthesis. With respect to factor (*c*), it was shown that during adolescence the leaves and roots together comprised 75 per cent of the plant; later the stems and finally the inflorescence grew rapidly and set up demands for large amounts of all essential nutrient elements. The patterns of accumulation and redistribution for nitrogen and phosphorus are shown in Figures 1 and 2, the data for phosphorus coming from Williams (54). With P_2 and P_3 all the nitrogen for the inflorescence came from other parts of the plant: with P_1 most, if not all the inflorescence nitrogen also came from other parts, and it was suggested for this treatment that withdrawal of phosphorus from the leaves may have caused protein breakdown, so that the leaves would provide a sufficient and closer source of nitrogen for the inflorescence. Williams (53) also gave evidence that there was competition between tillers for nitrogen. The absolute nitrogen content of the primary shoot in P_2 and P_3 suffered a temporary loss; resumption of intake by the primary shoot may have been partly from the medium, but also from vegetative tillers which died early. With P_1 this competition between tillers was apparently less severe, for the rate of intake of nitrogen by the primary shoot merely slowed down for a period prior to heading. Williams considered that the rapid death of noninflorescence-bearing tillers was due primarily to a withdrawal of nitrogen and phosphorus by inflorescence-bearing tillers.

As has been indicated, the interest in the tobacco work from Adelaide centers in the effects of prevention of inflorescence development. This treatment—the commercial practice of "topping"—was found by Petrie, Watson & Ward (32) to increase leaf and root weights considerably and stem weights only slightly. The increased leaf and root growth was the counterpart of the inflorescence in the untopped plants. The growth pattern of tobacco differs from that of cereals in that it shows continued increase in weight of roots and leaves during the time of active inflorescence development. The leaves were divided into four age groups, and Petrie *et al.* were able to show that the inflorescence had little effect on the growth of the oldest group, although it acted as a sink for its breakdown products during senescence. The inflorescence apparently limited the growth of the upper leaves by limiting increase in cell size, as was shown by Avery (2).

The diagrams of Figure 3, representing the intake of nitrogen and its progressive redistribution within the plant are reproduced from Watson & Petrie (49) for one only of their phosphorus treatments. Although topping brought about a significant increase in the dry weight of the whole plant, it did not affect the maximum nitrogen content. In considering Figure 3, it should be remembered that the stem fraction includes the continuously re-

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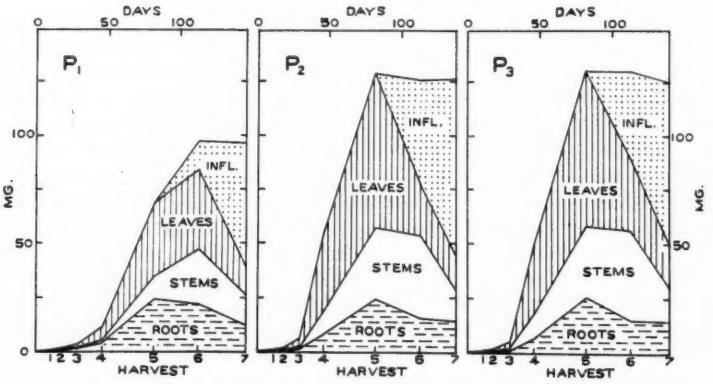

FIG. 1. The pattern of distribution and redistribution of nitrogen within the oat plant as affected by increasing phosphorus supply, P_1, P_2 and P_3. [after Williams (53) p. 79]

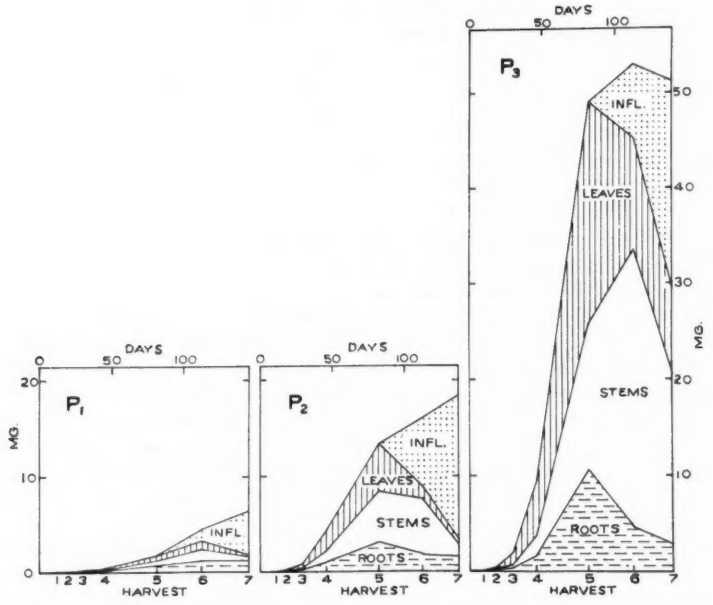

FIG. 2. The pattern of distribution and redistribution of phosphorus within the oat plant as affected by increasing phosphorus supply, P_1, P_2 and P_3. [after Williams (54) p. 338]

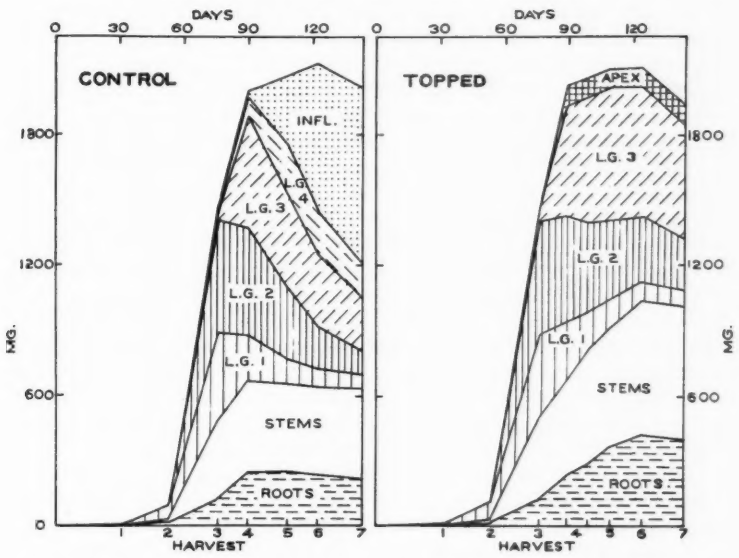

FIG. 3. The pattern of distribution and redistribution of nitrogen within the tobacco plant as affected by the removal of the inflorescence. L.G. = leaf group. [after Watson & Petrie (49) p. 315]

moved axillary shoots, and also the petioles and midribs of the leaves, which must still have been increasing in weight, at least in the upper leaves, until the end of the experiment. Extensive secondary thickening of stem and root is also a feature which contrasts with the growth pattern in cereals. There was no loss of nitrogen from the stems to the inflorescences in tobacco, as was found by Williams (53) in oats. Watson and Petrie suggest that the rise of competing sinks represented by organs higher in the acropetal series was an important factor leading to net export of nitrogen by the leaf groups. The first part of the nitrogen to be exported from the first group of leaves went mainly to the third group, and, in the untopped plants, net export from the second group started when intake was rapid by the fourth group of leaves and by the inflorescence. Most of the inflorescence nitrogen apparently came from the second and third leaf groups. In the topped plants there was a much greater accumulation and a smaller net loss of nitrogen from the leaf groups. Ultimately, however, there was a net export to the stems and roots, which suggests that these organs are potential sinks which become operative only if nitrogen is not wholly taken up by other parts. Watson & Petrie (49) found that increased phosphorus supply caused earlier senescence and more

rapid loss of nitrogen from the leaves as a whole, and in this their results resemble those of Williams (53). When considering possible mechanisms underlying their many examples of redistribution of nitrogen in the plant, Watson and Petrie favour the view that concentration gradients for certain soluble nitrogen compounds may be established between later-formed organs and the older leaves. They admit, however, that the younger organs may conceivably exert a hormonal influence on protein synthesis or hydrolysis in the older ones.

Walkley (47) and Walkley & Petrie (48) carried out experiments to determine whether there was an intrinsic factor superimposed on that of the external sinks in determining the net export of nitrogen from the mature and senescent leaf. Walkley sought to discover whether protein synthesis could occur in the fourth leaf of barley after it had attained maturity, and to do this she eliminated the external sinks as far as possible by removing all tillers and all younger leaves of the primary shoot. The effects of increasing the supply of nitrogen at three stages in the senescent decline of leaf four were studied, and it was shown that even leaves which had begun to yellow had not lost their capacity for protein synthesis. This result discounted the earlier conclusions of Pearsall & Billimoria (27, 28) who worked with detached leaves floated on nutrient solutions. These workers found that, in dicotyledons, protein synthesis occurred only in young immature leaves, and in Narcissus leaves only in the basal, elongating portion. McCalla (18) and Mothes (25) even suggested that protein synthesis could no longer occur even in attached leaves after a certain stage of growth. Results agreeing with those of Walkley were reported by Engel (11) for the attached first leaf of nitrogen-starved maize plants. Walkley (47) claims that her data can be satisfactorily explained on the assumption that net export of nitrogen from the leaves is mainly due to the presence of sinks, which causes an increased rate of translocation of soluble nitrogen compounds. There is a suggestion, however, that the rate at which protein can be synthesized declines progressively with age. Walkley & Petrie (48) examined the fifth leaf of barley in an experiment parallel to that of Walkley, but in more detail. In addition to protein and soluble nitrogen, data are given for asparagine, glutamine, residual amino, and ammonia nitrogen. In their discussion, the authors say that the main factor causing the net hydrolysis of proteins was evidently the removal of amino acids, which presumably follow concentration gradients set up between the fifth leaf and sinks in other parts of the plant. This is in keeping with the general picture which is emerging from this review, though there seems no reason, on the evidence, for selecting the amino acid fraction as the one involved in the gradient. From their studies on the transport of nitrogenous substances in the cotton plant, Maskell & Mason (19) favour their "residual" nitrogen fraction rather than amino acid or asparagine nitrogen as the important fraction in transport. Walkley and Petrie also found that the protein-amino acid relationship which held during protein hydrolysis

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varied with the past history of the leaf. They infer that the existence of such effects of treatment leave room for the operation of an intrinsic factor superimposed on that of the external sinks.

Although Tiver (43) and Tiver & Williams (44) present no direct information on the redistribution of mineral elements, they do make some observations relating to inherent differences in the growth patterns of flax, linseed, tobacco, wheat, oats, and Sudan grass. They stress the importance of differences in inflorescence weight ratio, and of the fact that secondary thickening occurs to varying extents in the stems and roots of dicotyledonous plants. Tiver and Williams also enunciate the principle that the distribution within the plant of carbohydrates and other metabolites is largely determined by the changing balance of demand set up by competitive meristematic tissues. This, of course, is implicit in much of the work reviewed above. They go on to state that the basic pattern of this dynamic balance is an inherent characteristic of the species or variety under consideration, but that the balance may be modified by many factors of the environment.

More recently, Williams (54) has further developed a concept of the integrated control of the movement of nitrogen and phosphorus within the plant, and his data for the intake and distribution of phosphorus in oats are reproduced as Figure 2 of this review. He considered the rate of intake of nitrogen and phosphorus to be governed by (*a*) the external concentration or supply of the nutrient, and (*b*) the demand for the nutrient set up by the growth and normal functioning of various plant parts, including the roots. He also considered that the influence of the external concentration of the nutrient on intake was more often subject to the controlling influence of the growth factors than otherwise. Each vegetative organ of a plant passes through phases of intake, relatively constant content, and of export of nitrogen and phosphorus. Each organ thus has a certain capacity to accumulate these nutrients, and in its senescent phase it constitutes a potential source for younger organs. The demand of each organ is usually met in part, if not entirely, by absorption from the medium, but the rate of intake by the roots is restricted to the extent that the nutrient is more readily available within the plant itself.

In support of these views Williams (54) points out that during the early development of the seedling, the grain was the sole source of phosphorus, and for some time it remained an important alternative source, especially when the external medium was deficient, as in his lowest treatment, P_1. The leaves and roots of the seedlings of this treatment derived all their phosphorus from the "stems" (which included the grain) until 18 days after sowing, and there was an indication of a loss to the phosphorus-deficient medium. With treatments P_2 and P_3, the "stem" fractions themselves showed net gains of phosphorus over the same period. During rapid vegetative growth, the roots, stems, and leaves all accumulated phosphorus rapidly, and the influence of the level of supply was very evident. Where there was a growth re-

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sponse to treatment (P_1 to P_2), however, Williams claims that the very great difference in phosphorus intake was due mainly to the greater growth (internal demand) and, therefore, only indirectly to the level of supply. This argument is based on rates of intake of phosphorus per unit increase in dry weight of the leaves, stems, and roots. These were at first little more than twice as great with P_2 as with P_1, and later they were actually less with P_2. Williams' further argument that the rate of intake of phosphorus when there was no longer a growth response (P_2 to P_3) was determined by the maximal capacities of the plant parts to accumulate the element, rather than by any direct effect of its concentration in the medium is likely to be less acceptable. The position can be clarified if the distinction is drawn between causation and explanation as has been done by Wilkie (50). In so far as the arguments used by Williams are deductions from the known properties of the system, they received explanation but not causal explanation. With respect to the intake of phosphorus by the plant as a whole in the present example, the only causal explanation that is admissible would seem to be in terms of the variation in external supply, and to that extent Williams seems to have made an unwarranted comparison between causal and functional explanations of the same phenomenon. What is really inferred is that the functional explanation is more meaningful than the strictly causal explanation. Wilkie defines a cause as some factor to which we attribute the disturbance of some regularity, and he points out that living things are extremely complex systems in which the parts have causal relations among themselves. The present discussion of the redistribution of mineral elements, with its emphasis on competition within the organism, is thus quite in keeping with this definition of causal explanation, but it does not take us very far toward an understanding of the ultimate regularity which is the living plant.

Reference to Figure 2 above shows that, with all treatments, phosphorus accumulated rapidly in the roots and leaves, then in the stems, and finally in the inflorescences, and that this was accomplished with varying degrees of redistribution of phosphorus. The inflorescence was an outstanding factor here, because of its very great demand for phosphorus. Thus, the percentages of the total plant phosphorus found in the inflorescences at maturity were 72, 82, and 43, for P_1, P_2, and P_3, respectively. By making certain reasonable assumptions, Williams reached the rather surprising conclusion that phosphorus-deficient oat plants derived only 30 per cent of their inflorescence phosphorus from other plant parts, whereas those with an excessive supply, P_3, derived no less than 93 per cent of their inflorescence phosphorus from these sources. For this last case, Williams claims that the external supply of phosphorus was still plentiful, but that an abundant and more accessible supply was made available by the senescent breakdown of the protoplasm in the leaves and roots, and later in the stems of these plants. Evidence already considered suggests that this breakdown was caused by the demand of the inflorescence for nitrogen. With phosphorus deficiency, P_1, on the other

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hand, the inflorescence phosphorus was more readily derived from the deficient medium than from the other plant parts.[2]

For the case of his phosphorus-deficient oat seedlings, Williams also examines some of the consequences of the interaction between redistribution and intake of phosphorus during the transition from the stage of dependence on seed reserves to full dependence on the medium. He found that seed reserves of phosphorus were exhausted 18 days after sowing, and that absolute leaf phosphorus had shown no increase 11 days later. Leaf growth did not cease, however, and nucleic acid phosphorus was reduced to one-fifth of the amount present in the leaves at the beginning of that period. During the same period, the whole of the phosphorus intake was retained by the roots, and the concurrent stimulation of root growth established the high root weight ratio which is so characteristic of phosphorus deficiency. The writer considers these observations to have importance relative to some aspects of morphogenesis, especially those which are subject to environmental influence. The balance between synthesis and hydrolysis of the nucleic acids appears to be very sensitive. In this connection Williams presents fortnightly values for amounts of protein nitrogen and for total and nucleic acid phosphorus in the leaves of *Phalaris tuberosa*. This work needs confirmation and further study, but the results suggest that unduly rapid shoot growth can accelerate the senescence of more mature tissues by inducing the hydrolysis of nucleic acids therein.

Mason & Phillis (23) cut off the supply of nitrogen to the roots of cotton plants in water culture and then followed the distribution of nitrogen over a period of four weeks. The experimental plants had been pruned of the lower, vegetative branches and to one leaf per fruiting branch. Separations were made into lower, upper, and apical regions, and roots, and into laminae, petioles, bark, and wood. Some nitrogen was lost altogether and some moved out of the fibrous roots and from the laminae and petioles of both lower and upper regions. Nitrogen moved into all fractions of the apical (juvenile) region and into the bark and wood of the stem and tap root. The effects can be interpreted as resulting from competition between the remaining meristems—apical and cambial—for nitrogen. The results are rather similar to those of Watson & Petrie (49) with "topped" tobacco plants (Fig. 3), though the roots as a whole lost nitrogen in the experiment with cotton. In the same paper, Mason & Phillis (23) showed that when bolls were left on the cotton plant, nitrogen migrated from vegetative to reproductive parts. Crowther (9) gave the distribution of nitrogen in the cotton plant at 15 stages of growth, but the picture is incomplete because the dead leaves could not be collected. More than half of the leaf nitrogen appears to have migrated from the leaves to the bolls, but there was no evidence of any migration from the stem.

[2] This experiment was conducted in sand culture and the P_1 plants got some of their phosphorus from the sand itself.

The Trinidad workers frequently used ringing as a means of interfering with the normal passage of carbohydrates and mineral elements from "source" to "sink," and in one such experiment for which absolute values are obtainable, Mason, Maskell & Phillis (22) isolated the basal region from the apical and root regions by appropriate rings in the bark. Export of nitrogen and phosphorus took place in the normal plants from the basal region via the phloem to the roots, and via the phloem or the xylem to the apical region. In the ringed plants the nitrogen which migrated from the leaves of the basal region accumulated in the bark and wood, but did not leave that region.

Sulphur.—In a review of sulphur metabolism, Wood (56) briefly discussed the redistribution of sulphur in plants and quoted evidence to show that sulphur is relatively immobile and does not leave the leaves as readily as does nitrogen. This is borne out by the characteristic symptoms of sulphur deficiency, where chlorosis tends to appear first in the upper leaves [Eaton (10) and others]. Nor do the lower leaves tend to dry up as in nitrogen deficiency. Barrien & Wood (4) examined the ontogenetic changes in protein and sulphate sulphur for the experiment with Sudan grass for which the nitrogen changes had been reported by Petrie (30). Net export of sulphur from the leaves was delayed and to a lesser extent than was net export of nitrogen. In this connection, Hanson, Barrien & Wood (14) concluded that there was a greater loss of cytoplasmic than of chloroplast protein during senescence, even though chlorophyll disappears from the leaves more rapidly than does protein. Wood & Barrien (57) also subjected ryegrass plants to a period of starvation in the dark. During the experimental period protein sulphur decreased in the leaves and sulphate sulphur increased. There was no evidence that sulphur was exported from the leaves under the conditions, and the authors suggest that this was due in part to the absence of any sink such as would be present with active growth in the light.

Quite recently, Kylin (17) studied the growth of deseeded wheat plants which, at a very early stage, were grown for three days in a complete nutrient solution containing radiosulphur, and then transferred to solutions with and without sulphur (not radioactive).

During the 21 days of the −S treatment there was no evidence of protein breakdown in the roots and protein nitrogen increased continuously in the shoots. Over the same period, however, protein sulphur rose to a maximum in the shoots and then decreased by 20 per cent. Kylin links this finding with that of Hanson, Barrien & Wood (14) on the high sulphur content of chloroplast protein, and implies that cytoplasmic protein built up, but chloroplast protein broke down in their sulphur-deficient shoots. This condition would, of course, be the reverse of that found by Hanson, Barrien and Wood, in plants with an adequate supply of sulphur. Kylin also showed a marked transport of radiosulphur from the shoots back into the roots as well as leakage into the solution, but his radioautographs show very little transport into later formed leaves. These facts, together with the tendency for sulphur de-

ficiency to favour root growth in this experiment, suggest that the sulphur demand of the root system is more powerful at this stage or that phloem transport to the roots is more efficient than that to the later formed leaves. The latter view gains some ground from the vascular connections shown by Boyd & Avery (5) for the wheat seedling. The midrib of the first leaf has no direct vascular connection with younger leaves, and it is conceivable that the timing of the interconnections between the lateral bundles of the second and first leaves was delayed sufficiently to enhance the advantage of the root with respect to phloem transport. The evidence is as yet rather slender, but the phenomenon is of some interest because it cannot very well have the same mechanism as that put forward by Williams (54) for the effect of phosphorus on the root weight ratio. In Kylin's experiment there was no sulphur in the −S medium to be preferentially used by the roots.

Potassium, calcium, and magnesium.—Direct quantitative evidence on the redistribution of these elements is scanty, and the most complete set of data for potassium and calcium is that of Wagner (46) for oats. The amount of potassium rose to an early maximum in the leaves, later in the stems, and still later in the inflorescence. Over 70 per cent of the leaf potassium was exported again and it is reasonable to suppose that it contributed first to the stem and then to the inflorescence. There was also an appreciable loss from the shoot as a whole. This confirmed an even greater loss of potassium found by Knowles & Watkin (16) from the tillers of wheat. Here, too, the potash of the ear appears to have come from the straw. Wagner (46) found calcium intake to be independent in leaf, stem, and inflorescence, with no evidence of redistribution. Mason & Maskell (20) and Petrie (29) briefly review the early work on the mobility of these elements, and provide theoretical discussion of the contrasting behavior of potassium and calcium with respect to redistribution. Willstätter (55) drew attention to the fact that magnesium was far in excess of calcium as a constituent of the ash of plant seeds, and this points to a greater mobility for magnesium. Other evidence pointing in this direction is the high diurnal fluctuations found by Phillis & Mason (34) for magnesium in cotton leaves, and the claim by Fudge (12) that developing citrus fruits can remove magnesium from nearby leaves and induce typical deficiency symptoms.

Minor elements.—From careful studies in water culture and with a copper-deficient soil, Piper (35) observed that numerous secondary tillers developed at the base of copper-deficient oat plants. This occurred with the cessation of growth of the original tillers and it is possible that there was a redistribution of copper under these conditions of extreme deficiency. Alternatively, the supply may have been insufficient to maintain growth in large tillers but sufficient to initiate secondary tillers. Piper also showed that small quantities of copper made available during early seedling stages enabled considerable growth to be made. Piper & Walkley (36) gave figures for the distribution of copper, zinc, manganese, and phosphorus between the grain and straw in

mature oat plants. Zinc and phosphorus were found to be concentrated in the grain and manganese in the straw, while copper was equally distributed between grain and straw. They conclude that zinc, like phosphorus, is translocated to the grain as maturity approaches, but that the translocation of copper is less complete. Although this was a reasonable interpretation of their data, Wood & Sibly (58) were fully justified in challenging it on the score that such deductions cannot be made from relative figures at final harvest. Wood & Womersley (59) made a study of the development and metabolism of copper-deficient oat plants on a calcareous sand from Robe, South Australia. They, too, observed secondary tillering in their deficient plants, and they attribute them to the partial suppression of the inflorescence. This suppression also produced ontogenetic changes similar to those produced by the "topping" of tobacco plants (see above). Wood and Womersley found very high values for the copper content of dead leaves, and their absolute data strongly suggest that copper is immobilized in the leaves and is not transported during senescence. These authors also found no evidence for any transport out of the stem, but they make the claim that copper must have been redistributed within the stem. This may have occurred, but the fact that there was an increase in dry weight over the interval does not, in itself, justify such a claim. Root copper could not be determined, but the evidence of Piper (35) was advanced in support of the roots as a possible source of inflorescence copper. Wood & Sibly (58) reached very similar conclusions with respect to the redistribution of zinc in the oat plant. No zinc was translocated from the leaves to other organs, but inflorescence zinc came from the roots and from the medium. Wood & Sibly also gave copper distribution data for one of their zinc experiments, and Figure 15 of their paper suggests quite strongly that some of the inflorescence copper came from the stem, though once again there is no evidence for migration from the leaves.

Williams & Moore (51) made a comprehensive study of the distribution of copper, zinc, manganese and molybdenum in oats grown on thirteen soils from different parts of Australia. Their findings for copper and zinc are in marked contrast to those of Wood *et al.* (58, 59). For copper their data indicate net export from the leaves for all soils,[3] and, on the average, this amounted to 30 per cent of that present at the penultimate harvest. The values ranged from 7 per cent to 58 per cent. Net export of stem copper was established for ten of the soils, but three showed continued intake until final harvest. Following the procedure of Williams (54), it may be concluded that all the inflorescence copper came from the leaves and stems on three of the soils, and more than 60 per cent came from these sources in four other cases. Williams & Moore (51) give a very similar picture for zinc, where the mean net export from the leaves was 34 per cent and all but two soils showed the

[3] The reviewer is indebted to Mr. C. H. Williams for permission to examine the results for the individual soils. In the original paper mean values only are presented.

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effect. By contrast, they found little evidence for the redistribution of manganese or molybdenum from the leaves, though export from the stems was the rule with manganese and sometimes occurred with molydbenum. Williams and Moore discuss at some length the discrepancy between their results for copper and zinc and those of Wood *et al.* For copper they specifically contrast their results with a calcareous sand from Yanchep, Western Australia, with those of Wood & Womersley (59) for the very similar soil from Robe, South Australia. They found no satisfactory explanation and could only point to the fact that different varieties of oats were used, and to the possibility of differences in cultural conditions. It is just possible that the release of copper from the leaves is dependent on protoplasmic disorganisation as a result of a relative shortage of some other nutrient element and it may be significant that Wood and Womersley supplied 9 essential elements in their basal dressing, whereas Williams and Moore supplied only nitrogen, phosphorus and, at a late stage, manganese in theirs. With respect to zinc, the latter authors produce evidence to show that Mulga oats has a much higher requirement than does Algerian oats, the variety used by themselves, and this gives point to their suggestion that the varietal differences could have been important.

Concluding remarks.—The general picture which emerges from this review is based very largely on correlative changes within the growing plant. In this connection Helder (15) expresses a note of caution when he says "one should be careful when interpreting the results of this type of experiments, which provide correlations rather than direct causal relations." This would be said even more emphatically by those who insist on a strictly analytic approach in experimental biology. The value of a search for correlation (integration would be a better term) resides in its emphasis on relations within the organism as a whole, and in its capacity to throw up problems for solution by more orthodox procedures. It is suggested that far more caution is necessary when using results from abstract procedures such as tissue culture for the interpretation of growth processes within the intact plant. Helder has, of course, used the intact plant for his very neat demonstration that the intake of nitrate is regulated by growth in the sense of utilization as a result of synthetic processes. It is significant that this gives direct experimental sanction to conclusions arrived at previously by the methods of correlation [Watson & Petrie (49), Williams (54)]. Then, too, the findings of Russell & Martin (38) on the distribution of phosphorus between root and shoot provide a more detailed analysis of phenomena concerning which concordant conclusions had been drawn from the correlative study of growth and net intake alone [Williams (54)].

There is an increasing interest in the use of the intact plant for studies of nutrition and ion intake. Some examples of more than passing interest are papers by Steward, Prevot & Harrison (41), Alberda (1), van Andel, Arisz & Helder (45), Helder (15), Stout *et al.* (42), Butler (7), Russell & Martin (38)

and Brouwer (6). Such studies have already brought to light some interesting interrelations between growth and metabolism, and it is to be hoped that they will be extended to other aspects of metabolism. As Petrie (30) has stated,

"the growth and development of an organism is the result of a number of ontogenetic processes, among which complex interrelations exist. The interpretation of these interrelations, and of the manner in which these processes are integrated to produce the living plant is the fundamental problem in the study of growth."

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NITROGEN METABOLISM[1,2]

By George C. Webster[3]

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

The two years that have elapsed since the appearance of the last review on this subject (1) have witnessed a substantial increase in our knowledge of the detailed biochemical pathways involved in nitrogen metabolism in plants. In addition, considerable information has accumulated on (*a*) the identification of hitherto unsuspected nitrogenous plant constituents; (*b*) the characterization of plant proteins; and (*c*) the nitrogen nutrition of plants, including studies on nitrogen fixation. These developments have been accompanied by the appearance of several interesting and important reviews. Of these, two new volumes from the McCollum-Pratt Institute must be singled out for special commendation. These reports of symposia on *The Mechanism of Enzyme Action* (2) and on *Amino Acid Metabolism* (3) continue the high standards of previous works and deserve the attention of all plant physiologists. Both contain provocative discussions on several aspects of nitrogen metabolism. In addition, singularly clear and comprehensive reviews on amino acid metabolism [Meister (4); Cohen (5); Greenberg (6)], on protein synthesis in animals [Borsook (7)] and in microorganisms [Gale (8)], and on the general topic of plant protein metabolism [Steward (9)] have appeared.

It is obviously impossible to discuss adequately all of the recent advances in plant nitrogen metabolism. The author has, therefore, chosen for discussion four subjects of investigation in which he feels either that considerable progress has been made or that the outlook for future development is especially bright. These topics are (*a*) nitrate reduction, (*b*) amino acid metabolism, (*c*) amide and peptide bond synthesis, and (*d*) protein synthesis.

Nitrate Reduction

The simple fact that nitrate is transformed in the plant to ammonia has been known for a long time. From purely theoretical considerations, the sim-

[1] The survey of the literature pertaining to this review was concluded in October, 1954.,

[2] The following abbreviations will be used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; TPN^+, triphosphopyridinenucleotide; TPNH, reduced triphosphopyridinenucleotide; DPN^+, diphosphopyridinenucleotide; DPNH, reduced diphosphopyridinenucleotide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PCMB, *p*-chloromercuribenzoate; CoA, coenzyme A; Glu, glutamate; Glu-NH_2, glutamine; Glu-NHOH, glutamyl hydroxamate; Cys, cysteine; P_i, orthophosphate; DNA, desoxyribonucleic acid; RNA, ribonucleic acid, E, enzyme.

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plest and most direct pathway of reduction consistent with known mechanisms of enzymatic hydrogen transfer would involve the intermediate formation of nitrite, hyponitrite, and hydroxylamine. The thought that nitrate reduction may proceed through some such intermediate steps has permeated the literature for many years, but, until recently, little progress was made in verifying this hypothesis. In general, research in the past was of necessity directed toward the attempted isolation of one or the other of the suspected intermediates. The claims of various investigators to have detected such substances have already been reviewed by Wood (1). Not unexpectedly, the results in almost all cases are somewhat equivocal. The high reactivities of nitrite, hyponitrite, and hydroxylamine make it very unlikely that these substances will ordinarily accumulate to chemically detectable levels in the living cell. Recent work (2) on enzyme-bound intermediates suggests the possibility that nitrate reduction might occur at rapid rates in a biological system without any accumulation of free intermediate products. It is not surprising, therefore, that attempts to isolate the postulated reduction intermediates from living cells have given such disappointing results.

The work of Stickland and others (10, 11, 12), however, had shown that not only are bacterial cell suspensions capable of reducing nitrate to nitrite, but also that cell-free extracts which carry out this reduction can be prepared. Although this evidence suggested the possible occurrence of a similar nitrate reduction reaction in plants, earlier attempts with cell-free plant extracts (13) were not successful because reduction was detected only after long incubation periods (24 hr.). Since these incubation periods also consistently result in the growth of nitrate reducing bacteria in plant extracts (1), the earlier experiments were of value principally in demonstrating that the plant reductase must be considerably different in its properties from the bacterial reductase.

Nitrate reductase.—Evans & Nason (14, 15, 16), in an outstanding piece of work, have isolated and characterized enzymes from both soybean leaves and *Neurospora* which unequivocally catalyze a pyridine-nucleotide-dependent conversion of nitrate to nitrite. This and subsequent work of the McCollum-Pratt group leaves no doubt that the reduction of nitrate to nitrite does occur readily in plants. The reductase from both sources catalyzes the following overall reaction:

$$NO_3^- + TPNH + H^+ \rightleftarrows NO_2^- + TPN^+ + H_2O.$$

While the *Neuropsora* enzyme is relatively specific for TPNH, the soybean enzyme can use $TPNH^2$ and $DPNH^2$ equally well. This is the major difference between the two enzymes which has appeared thus far. Each has been purified some sixty to seventyfold and the overall properties of the enzymes determined. Reductase activity was found in the tissues of six other plant species examined in addition to soybean leaves. More recently, Evans (17) has described a highly specific DPNH-nitrate reductase from soybean nodules. It seems likely from these findings that a pyridine-nucleotide-dependent nitrate reductase is of general occurrence in higher plants.

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Aside from its great potential importance to the cellular conversion of nitrate to ammonia, the nitrate reductase itself has many properties of interest. The marked sensitivity of the enzyme to *p*-chloromercuribenzoate, which, however, can be alleviated with cysteine or glutathione, indicates the necessity of sulfhydryl groups for activity. Likewise, reactivation studies, fluorimetric analysis, and other investigations (15, 16) suggest that the enzyme is a flavoprotein with flavin adenine dinucleotide as the prosthetic group. The involvement of a metal in reductase activity was indicated in the earlier work by the strong inhibition of activity obtained with cyanide, azide, thiourea, *o*-phenanthroline, and 8-hydroxyquinoline. Nicholas, Nason & McElroy (18, 19, 20) have now identified the metal constituent as molybdenum. By assaying reductase preparations obtained from *Neurospora* grown on media deficient in various micronutrients, it was found that molybdenum deficiency alone resulted in a marked decrease in enzyme activity. In addition, enzyme preparations which had been inactivated by dialysis against cyanide could be reactivated only by molybdenum. Furthermore, it was found that the molybdenum content paralleled the specific activity of the enzyme during fractionation of the enzyme. The accumulation of nitrate in plants suffering from molybdenum deficiency (21 to 24) appears, therefore, to be a direct reflection of decreased nitrate reductase activity caused by deficiency of the essential metal constituent. The exact mechanism by which molybdenum acts in nitrate reduction is not completely clear. McElroy & Nason (25) suggest that it may act as an electron carrier between reduced FAD[2] and nitrate as follows:

$$\text{TPNH} \rightarrow \text{FAD} \rightarrow \text{Mo}^{-} \rightarrow \text{NO}_3^{-}.$$

Evidence in favor of this sequence has been provided by Nicholas & Nason (26). They find that the molybdenum-free enzyme has lost its ability to catalyze the reduction of nitrate by TPNH or reduced FAD, but will catalyze the reduction of FAD by TPNH. The addition of Na_2MoO_4 restores the ability of the molybdenum-free enzyme to catalyze the reduction of 2, 6-dichlorophenol indophenol by either reduced FAD or TPNH. Conversely, the enzymatic reduction of nitrate by reduced 2, 6-dichlorophenol indophenol is cyanide-sensitive, does not require FAD, and requires molybednum. That the molybednum acts as an electron carrier is also indicated by the ability of chemically reduced molybdenum to serve as an electron donor for enzymatic nitrate reduction in the absence of TPNH and reduced FAD.

In addition to the above evidence for the postulated reaction sequence, Nicholas & Nason (26) find that the sulfhydryl groups which are necessary for nitrate reductase activity appear to be chiefly concerned with the reduction of FAD by TPNH. Low concentrations of PCMB[2] inhibit this step by 75 per cent, but inhibit the other steps only *circa* 25 per cent. They suggest that the sulfhydryl group either functions as an electron carrier or is involved in binding of TPNH or of FAD to the enzyme.

Nitrite reduction.—In connection with their studies on nitrate reductase, Evans & Nason (15) showed that nitrite disappears (although slowly) in the

presence of homogenates of soybean leaves and TPNH. The reaction is enzymatic and has an absolute dependence upon TPNH. This nitrite reductase activity is inhibited some 80 per cent by 0.001 *M* hydroxylamine. A similar system was found in tomato root homogenates.

Vanecko (27) likewise studied the reduction of nitrite using leaf sections of wheat, corn, and grass. Nitrite disappearance could be demonstrated to proceed in both light and darkness, although the rate in light was 10 to 20 times more rapid. By the use of N^{15}-nitrite, it was demonstrated that nitrite-nitrogen is incorporated into the amino nitrogen of both ethanol-soluble and insoluble fractions. This indicates that the reduction of nitrite continues to the level of ammonia. The stoichiometry of nitrite reduction in the light (with photochemical splitting of H_2O providing the reducing power) was found to be in accordance with the formulation:

$$2HNO_2^- + 2H_2O \rightleftarrows 2NH_3 + 3O_2.$$

The demonstration of enzyme systems in both soybean leaves and *Neurospora* that carry out the conversion of nitrite to ammonia has been reported by Nason, Abraham & Averbach (28). The enzymes have been purified some ten to fifteenfold and can utilize either DPNH or TPNH. For each mole of nitrite reduced, three moles of DPNH are oxidized. The enzymes from both sources are apparently metallo-flavoproteins. Hydroxylamine is evidently an intermediate in the reduction sequence.

Hydroxylamine reductase.—In view of the demonstrated reduction of nitrite, it is significant, therefore, that Zucker & Nason (29) have isolated an enzyme from *Neurospora* extracts that catalyzes the reduction of hydroxylamine to ammonia. The enzyme has been purified about tenfold and partially characterized. Rate of reduction with TPNH is about double that with DPNH. Measurements of DPNH disappearance, DPN production[2], and ammonia production suggest that the reaction proceeds as follows:

$$NH_2OH + DPNH + H^+ \rightleftarrows NH_3 + DPN^+ + H_2O,$$

and it is thus very similar to nitrate reduction.

Hydroxylamine reductase, like nitrate reductase, is sensitive to *p*-chloromercuribenzoate and to metal-binding agents. It is particularly sensitive to cyanide, being inhibited 50 per cent by 1×10^{-6} *M* HCN. The reductase activity is increased by added FAD but not by FMN.[2] This suggests that the enzyme, like nitrate reductase, is a metallo-flavoprotein. The nature of the metal involved is not known, although preliminary experiments (30) have indicated that it is probably not molybdenum. The discovery of a hydroxylamine reductase, when viewed together with the information concerning the nitrate and nitrite reductase suggests that the familiar stepwise scheme for conversion of nitrate to ammonia may indeed be correct.

$$\text{Nitrate} \xrightarrow{\text{Nitrate reductase}} \text{Nitrite} \xrightarrow{\text{Nitrite reductase}} \text{Intermediate} \rightarrow$$

$$\text{Hydroxylamine} \xrightarrow{\text{Hydroxylamine reductase}} \text{Ammonia}$$

The existence of an intermediate in the above scheme at the reduction level of hyponitrite is suggested both by the results of Nason *et al.* (28) and by our present knowledge of hydrogen transfer mechanisms in living systems.

The possibility of alternate pathways of hydroxylamine reduction must be recognized, however. One such pathway, suggested by Silver & McElroy (31), involves the reaction of hydroxylamine with pyridoxal phosphate (B_6-P), followed by reduction of the oxime to pyridoxamine phosphate (B_6-P-NH_2). Pyridoxamine phosphate is then visualized as transaminating with an α-keto acid to form an amino acid with regeneration of pyridoxal phosphate:

$$B_6\text{-P} + NH_2OH \rightarrow B_6\text{-P-oxime} \rightarrow B_6\text{-P-}NH_2$$
$$B_6\text{-P-}NH_2 + \alpha\text{-keto acid} \rightleftarrows B_6\text{-P} + \text{amino acid}$$

The authors (31) point out that pyridoxal apparently participates in nitrite reduction, and in view of our present knowledge of the general transaminating ability of pyridoxamine phosphate, the hypothesis is certainly worth attention.

The importance of molybdenum to nitrate reduction raises a question of the potential importance of this micronutrient metal to other essential enzymes in plants. That molybednum is in fact important in other systems is suggested by the finding of Hewitt & McCready (32) that molybdenum is essential for tomatoes, regardless of whether or not nitrogen is given as nitrate or in other forms including ammonia. Only the relative molybdenum requirement is dependent upon the nitrogen source. It is known that molybdenum is a constituent of xanthine oxidase (33) and possibly of hydrogenase (34). Of particular interest in regard to nitrogen metabolism is the observation of Nicholas *et al.* (19, 20) that molybdenum deficiency decreases not only the nitrate reductase activity, but also the glutamic dehydrogenase activity of *Neurospora*. It is not clear as yet whether or not this decrease is a reflection of a requirement of molybdenum for glutamic dehydrogenase formation or whether molybdenum may be an integral part of the dehydrogenase itself. The latter possibility is worthy of serious consideration in view of our rapidly increasing knowledge of metals (and especially metallo-flavoproteins) in enzyme systems concerned with electron transport (35).

Amino Acid Metabolism

Although much valuable information was obtained from early work in which amino acid and protein levels were measured under a variety of conditions, it was not until isotopes became available that real insight began to be gained into the myriad of transformations which amino acids undergo in living cells. Early experiments with N^{15}-ammonia established that the nitrogenous constituents of plants (particularly amino acids) undergo constant breakdown and resynthesis. Thus, Vickery *et al.* (36) reported that the nitrogen of N^{15}-ammonia is rapidly assimilated by growing tobacco plants into amides, amino acids, and proteins. The quantity of isotope incorporated was greater than might be expected as a result of growth alone, and the authors concluded that the nitrogenous constituents of plants are in a dynamic state similar to that found by Schoenheimer in animals (37). Similar findings were reported by

Hevesy *et al.* (38) with sunflower plants. These experiments were extended by MacVicar & Burris (39), who showed that glutamic and aspartic acids in particular become rapidly and highly labeled with N^{15} after plants are supplied with N^{15}-ammonia. MacVicar & Burris suggest that the transformation of α-ketoglutarate and oxalacetate to glutamate and aspartate must be very rapid. This is in agreement with the findings of Rautanen (40) who showed that these dicarboxylic amino acids, their amides, and alanine are the primary products formed during the incorporation of ammonia into the organic nitrogen fraction in plants. All of these results are in accord with the now well-established view that the ammonia assimilated by a plant either directly or through nitrate reduction is incorporated into cellular metabolic pathways chiefly through the amination of α-ketoglutarate, oxalacetate, and pyruvate. The three amino acids, glutamate, aspartate, and alanine, therefore, occupy key positions in amino acid metabolism.

The advent of radioactive carbon 14 allowed a new approach to amino acid formation to be made. Not only the turnover of N^{15}-ammonia, but also the formation of the actual carbon skeletons of the amino acids could be investigated with the aid of the carbon isotope. Furthermore, where studies with N^{15} require milligrams of experimental material, studies can easily be carried out with microgram quantities of C^{14}-labeled compounds. The ease of assay of C^{14} has undoubtedly been in part responsible for the progress in elucidation of metabolic pathways which has occurred in recent years. Studies on amino acid formation in plants with radioactive carbon have chiefly followed two lines of investigation. The first concerns the transformation of various metabolites (glucose, acetate, pyruvate, carbon dioxide, urea, etc.) into amino acids. These studies have utilized either nonphotosynthetic cells (yeast, bacteria, root tissue), or photosynthetic tissues under conditions of darkness. The second line of investigation has been concerned entirely with the incorporation of CO_2 into amino acids as a result of photosynthesis. Although early studies along this latter line were obviously only by-products of the more tantalizing investigation on photosynthetic production of carbohydrate, more recent investigations have been concerned chiefly with amino acid synthesis during photosynthesis.

Nonphotosynthetic amino acid formation.—Rogers (41) has carried on a kinetic study of the conversion of radioactive acetate and sucrose into amino acids by leaves and roots of bean seedlings. Uniformly labeled sucrose was initially transformed most rapidly by leaves into alanine, glutamate, and aspartate. Moderate rates of C^{14} incorporation into glycine, threonine, phenylalanine, valine, and methionine were observed. The finding of an initial rapid labeling of alanine is consistent with the view that this amino acid arises from pyruvate which in turn is produced from sucrose through glycolysis. The high rates of labeling of glutamate and aspartate are likewise consistent with the transformation of pyruvate to Krebs cycle intermediates and the subsequent conversion of α-ketoglutarate and oxalacetate to glutamate and aspartate. This conclusion is supported by the further finding of Rogers (41) that acetate likewise is incorporated rapidly into glutamate and aspartate and more slowly into

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other amino acids. Those acids which are probably most closely related metabolically to the dicarboxylic acids are labeled at rates closest to those of glutamate and aspartate. The rapid labeling of the dicarboxylic acids is exactly what would be expected if acetate undergoes assimilation by an initial activation to form acetyl-CoA[2], followed by the condensation of acetyl-CoA and oxalacetate to form citrate, and subsequent metabolism of citrate by way of the Krebs cycle. That acetate is converted to acetyl-CoA in plants has been demonstrated by Millerd & Bonner (42). The operation of the Krebs cycle in plants is likewise well established (43, 44). The rapid formation of glutamate, aspartate, and alanine in these experiments is thus consistent with the earlier cited evidence of MacVicar & Burris (39) that glutamate and aspartate undergo rapid turnover in plants, and reaffirms the central metabolic position of these amino acids. It is interesting to note that Rogers finds that acetate is incorporated into leucine and valine relatively slowly in bean plants. This is in contrast to the finding of Arreguin, Bonner & Wood (45) that in guayule seedlings acetate is incorporated into leucine and valine in greater amounts than into any other substance examined. It would appear that a plant engaged in isoprenoid synthesis (as guayule) is particularly well-adapted to form other, closely related branched-chain compounds.

Racusen & Aronoff (46) have studied the incorporation of $C^{14}O_2$ into the amino acids of excised soybean leaves in the dark. The greatest amount of radioactivity was found to be incorporated into arginine, followed by glutamate, aspartate, asparagine, serine, glycine, and alanine. These findings are similar to those obtained by Anfinsen, Beloff & Solomon (47) who fed $C^{14}O_2$ to liver slices, and by Abelson, Bolton & Aldous (48) who fed $C^{14}O_2$ to bacteria. All of these results with the exception of those concerned with arginine can be explained by known carboxylation reactions or other transformations, and are in agreement with the pathways indicated by Rogers' experiments. Racusen & Aronoff (46) degraded the arginine produced during their experiments and found 75 per cent of the total radioactivity of the amino acid in the guanido carbon. This is in agreement with the pattern of labeling of arginine in liver (47) and in bacteria (48). In these organisms the guanido labeling of arginine after $C^{14}O_2$ feeding has been interpreted as resulting from the operation of an ornithine cycle. Racusen & Aronoff suggest that such a cycle may also operate in soybean leaves. Further evidence for the operation of at least a modified ornithine cycle is discussed further on in this review.

Actually, the results of both Racusen & Aronoff (46) and Rogers (41) are qualitatively so similar to many of the recent findings of investigators on carbon 14 incorporation into amino acids of microorganisms (49, 50, 51) that it would appear that the pathways of formation of amino acids in higher plants may be very much like those in certain microorganisms. In investigations of the detailed chemical pathways of amino acid formation, therefore, the work already carried out on microorganisms should prove to be a valuable guide for studies on both higher plants and animals. An example of this is provided by recent bacterial studies. Rogers (41) finds that many amino acids in higher plants

become labeled after glutamate, aspartate, and alanine are already highly labeled. This suggests that certain amino acids may be formed from these three key amino acids. The pathways by which such transformations may come about have been extensively investigated with mutants of *Neuropsora*, *Escherichia coli*, and other microorganisms (3, 6). Using the "isotope competition" method of analysis, Abelson (52) has summarized evidence for a number of the suspected metabolic relationships between amino acids in *E. coli*. The results indicate that the following sequences may be operative:

(*a*) Glutamate → N-acetylglutamate → N-acetylglutamic-γ-semialdehyde → N-acetylornithine → ornithine → citrulline → arginine
(*b*) Glutamate → glutamic-γ-semialdehyde → Δ-pyrroline-5-carboxylic acid → proline
(*c*) Aspartate → lysine
(*d*) Aspartate → homoserine → methionine
(*e*) Aspartate → homoserine → threonine → α-ketobutyrate → α-β-dihydroxy-β-methylvalerate → α-keto-β-methylvalerate → isoleucine
(*f*) Pyruvate → alanine
(*g*) Pyruvate → α-β-dihydroxyisovalerate → α-ketoisovalerate → α-ketoisocaproate → leucine
(*h*) α-ketoisovalerate → valine
(*i*) Serine → glycine; serine → cysteine; serine → pyruvate; threonine → glycine; phenylpyruvate → phenylalanine.

That certain of the pathways suggested above are actually operative has been demonstrated by the isolation and characterization of the enzymes involved. Thus, the results of Abelson (52) suggest a synthetic relation between aspartate, homoserine, threonine, and α-aminobutyrate. Black & Gray (53) have isolated an enzyme from yeast that catalyzes the phosphorylation of aspartate by the following reaction:

$$\text{Aspartate} + \text{ATP}^{2} \rightleftarrows \text{aspartyl phosphate} + \text{ADP}^{2}.$$

An additional enzyme system converts the aspartyl phosphate to homoserine (54). Black has also indicated that evidence has been obtained for the enzymatic conversion of homoserine to threonine (55). The further transformation of threonine to α-aminobutyrate has been reported by Lien & Greenberg (56). It can thus be seen that pathways suggested by isotope competition can be confirmed enzymatically. It can be anticipated that further studies along these lines will substantially increase our understanding of the chemical pathways involved in amino acid synthesis.

Amino acid formation during photosynthesis.—The photosynthetic formation of amino acids is actually only a special case of the more general problem of amino acid synthesis in plants. The pattern of $C^{14}O_2$ incorporation into amino acids during short periods of photosynthesis is considerably different from that in the dark or in nonphotosynthetic tissues. This difference, however, is only a reflection of the labeling of the respective carbon skeletons in the early stages of photosynthesis. Thus, the early work of Stepka and co-workers (57) showed that not only phosphoglycerate and malate, but also alanine and aspartate are labeled after only 30 sec. of photosynthesis. In somewhat longer periods of photosynthesis (90 sec. and more) serine and glycine also become labeled. Similar results have been reported by Vernon & Aronoff (58) who found that soybean

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leaves rapidly incorporate $C^{14}O_2$ into alanine, serine, and glycine, but not into aspartate. Degradation of these amino acids shows that the major portion of the radioactivity in both alanine and serine reside in the carboxyl carbon, while the carbons of glycine are about equally labeled. This labeling suggests that alanine arises from phosphoglycerate, and that serine and glycine are formed from alanine, presumably by some such pathway as

$$\text{alanine} \rightarrow \text{serine} \rightarrow \text{ethanolamine} \rightarrow \text{glycolate} \rightarrow \text{glyoxylate} \rightarrow \text{glycine.}$$

Unfortunately, the detailed relations between these amino acids have not as yet been elucidated. Although the manner in which malate arises during the early stages of photosynthesis is likewise somewhat obscure, the transformation of this acid to aspartate (probably via oxalacetate) seems quite probable.

As the photosynthetic period becomes longer, the number of amino acids which have become detectably labeled increases considerably. The pattern of labeling becomes less unique and begins to resemble that obtained with C^{14}-glucose. Racusen & Aronoff (46) have determined the incorporation of $C^{14}O_2$ into soybean leaf amino acids after 1 hr. of photosynthesis. They find considerable activity in aspartate, asparagine, glutamate, glycine, serine, and alanine. Small amounts of activity were also found in phenylalanine, tyrosine, proline, leucine, and arginine. Degradation of phenylalanine and of leucine showed that they had become uniformly labeled, indicating that the entire synthesis must take place during the photosynthetic period. The production of branched-chain and aromatic amino acids occurs most rapidly in young leaves and slows up markedly after maturation. It appears, therefore, that even in relatively short periods of photosynthesis, CO_2 can be incorporated into most amino acids.

Schieler, McClure & Dunn (59) have studied the incorporation of $C^{14}O_2$ into the amino acids of *Chlorella* during a three-day period of photosynthesis. They find that all amino acids examined show some radioactivity. Very high specific activities were found in aspartate, arginine, phenylalanine, glutamate, and histidine. Alanine, which is so rapidly labeled in the initial stages of photosynthesis, showed only a moderate activity, while serine and glycine were also relatively low in activity. With this long period of photosynthesis, labeling is qualitatively so similar to that obtained from glucose-C^{14} incorporation in the dark that almost all novelty resulting from the effect of light has disappeared. One noteworthy result, however, was the high activity found in phenylalanine as compared with the low activity of tyrosine. This difference, which has also been observed during sucrose-C^{14} feeding in the dark by Rogers (41), has caused Schieler *et al.* (59) to suggest that these closely related amino acids may possibly be formed by different pathways. Another interpretation may be made, however. Udenfriend & Cooper (60) have shown that phenylalanine is enzymatically converted to tyrosine. It seems possible that the high activity in phenylalanine and low activity in tyrosine might be merely a reflection of a very low capacity for this oxidative step. In addition to the above studies on amino acid synthesis, considerable information has accumulated on certain aspects of the chemical pathways involved in amino acid metabolism.

Amino acid oxidation.—Rogers (61) has demonstrated the oxidation of a

series of L-amino acids by squash preparations. The system catalyzes a slow but measurable oxidation of many of the common amino acids, the highest rates being obtained with alanine, arginine, cysteine, isoleucine, ornithine, α-aminobutyric acid, cysteic acid, and γ-aminobutyric acid.

Amino acid decarboxylation.—Rogers (61) has also examined the anaerobic decarboxylation of amino acids. With squash preparations, he found evidence for the rapid decarboxylation of only one amino acid, glutamate. Glutamic decarboxylase, which has been studied extensively by several investigators [Okunuki (62); Schales *et al.* (63); Beevers (64); Miettinen & Virtanen (65)], apparently occupies a unique place in the metabolism of amino acids in plants. Aspartate was decarboxylated by the squash preparation at a rate of about one-hundredth that of glutamate. Of other amino acids, only cysteic was decarboxylated at a readily measurable rate. Fowden (66) has reported that the decarboxylation of α-methyleneglutamate by extracts of barley roots and tulip leaves is probably catalyzed by the well-known glutamic decarboxylase. Whether or not this enzyme is also responsible for the slow aspartate decarboxylation observed by Rogers is still uncertain.

In contrast to their negligible anaerobic decarboxylative activity [Rogers (61)], squash extracts decarboxylate most amino acids under aerobic conditions. The rough parallelism between rate of oxidation of an amino acid and its rate of oxidative decarboxylation suggests that the amino acids may be first oxidatively deaminated, followed by decarboxylation of the keto acid:

$$\text{Amino acid} \xrightarrow[-NH_3]{+\frac{1}{2}O_2} \alpha\text{-keto acid} \xrightarrow{-CO_2} \text{fatty acid.}$$

The rates of these aerobic decarboxylations, however, are relatively slow and are apparently limited by the deamination step.

Transamination.—The sluggish nature of oxidative deamination in plants suggests that removal of α-amino groups may occur more generally by transfer of this group to a keto acid rather than by its outright oxidative removal. The widespread occurrence of transamination systems would tend to support such a view. Early work established that transamination systems involving glutamate, aspartate, and alanine (and their keto analogues) are widely distributed in plants [Kritzman (67)]; Virtanen & Laine (68); Albaum & Cohen (69); Leonard & Burris (70)]. The surprisingly wide scope of transamination reactions has been realized only recently, however. Stumpf (71) extended the list of amino acids involved in transamination to include α-aminobutyrate, leucine, isoleucine, valine, and norvaline. In addition to these, Fowden & Done (72) demonstrated transamination between α-ketoglutarate and γ-methyleneglutamate, γ-methylglutamate, and glycine. Miettinen & Virtanen (73) showed that both γ-aminobutyrate and citrulline can transaminate with α-ketoglutarate in the presence of homogenates of peas or alder. Finally, Wilson King & Burris (74), in a comprehensive study, demonstrated transamination between 17 amino acids and α-ketoglutarate, thus showing conclusively that transamination is a general reaction in plants and involves most of the known amino

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acids. They likewise confirmed the wide distribution of transamination systems in plant tissues. In agreement with the results of Rogers (61) on oxidative deamination, Wilson *et al.* (74) find that reductive amination occurs only very slowly in plant tissues. The occurrence of a variety of keto acids in plants (75) suggests that amino acid synthesis and degradation may take place chiefly through transamination reactions.

The probable major importance of transamination reactions to amino acid metabolism makes the mechanism of these reactions of particular interest to the physiologist. It is well known that the activity of transamination systems of a variety of organisms depends upon pyridoxal phosphate. Metzler & Snell (76) have demonstrated conclusively that transamination reactions of the following type,

$$\text{Pyridoxal} + \alpha\text{-amino acid} \rightleftarrows \text{pyridoxamine} + \alpha\text{-keto acid},$$

proceed nonenzymatically in aqueous solution at 100°C. and pH 3 to 8 in the presence of metal ions. They conclude (77) that the "catalytic potentialities of pyridoxal phosphate enzymes are, to an exaggerated degree, those of their prosthetic group, and that the nonenzymatic and enzymatic reactions proceed by closely similar mechanisms." An investigation of the structural features of pyridoxal necessary for activity has led to the conclusion that the nonenzymatic transamination reaction proceeds through the intermediate formation of a Schiff base as depicted in Figure 1. The enzymatic reaction presumably also proceeds by the intermediate formation of a similar Schiff base. Two observations must be explained, however, before findings with the enzymatic and nonenzymatic reactions can be completely integrated. First, the nonenzymatic reaction has a metal ion requirement that has apparently never been observed in the enzymatic reaction. It may be that a metal requirement will become evident when transamination enzymes have been purified more highly. Conversely, it is possible that the enzyme itself may act in place of the metal. Secondly, the

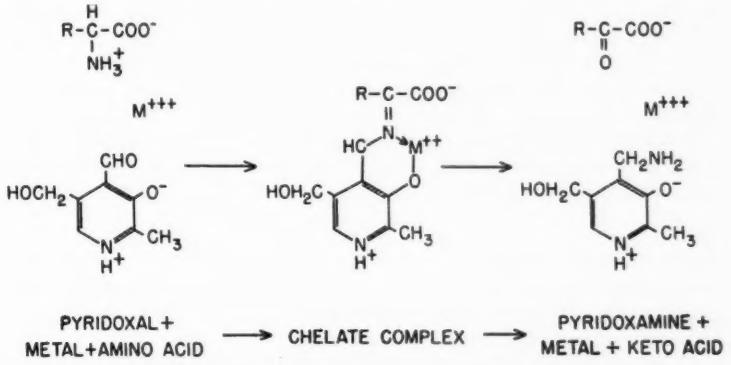

FIG. 1. Formation of chelate complex during nonenzymatic transamination.

requirement for the phosphorylated form of pyridoxal in the enzymatic reaction does not hold in the nonenzymatic reaction. Both Meister (78) and Metzler *et al.* (77) have proposed that the phosphate group is involved in the linkage of the cofactor to the enzyme. Meister (78) has formulated enzymatic transamination in this manner:

$$\text{Enzyme} + \text{pyridoxal phosphate} \rightleftarrows \text{enzyme} - \text{pyridoxal phosphate}$$
$$(\text{or pyridoxamine phosphate}) \qquad (\text{or enzyme} - \text{pyridoxamine phosphate})$$
$$\text{Enzyme} - \text{pyridoxal phosphate} + \text{glutamate} \rightleftarrows \text{enzyme} - \text{pyridoxamine phosphate} + \alpha\text{-ketoglutarate.}$$

The first reaction above may be regarded as the constitution of the complete transaminase from apoenzyme and prosthetic group. The second depicts the transamination itself in which pyridoxal phosphate acts as an amino group acceptor and pyridoxamine phosphate as an amino group donor.

Urea metabolism.—The increasing importance of the foliar application of urea in the nitrogen nutrition of agriculturally important plants makes it imperative to have further knowledge of urea metabolism in the plant. It is, therefore, worthwhile to discuss what is known of the relationship of urea to other nitrogenous substances. In mammalian tissues, the metabolism of urea is intimately linked to the amino acids, ornithine, citrulline, and arginine by means of the urea cycle:

$$\text{ornithine} + CO_2 + NH_3 \rightarrow \text{citrulline} \rightarrow \text{arginosuccinate}$$
$$\text{arginosuccinate} \rightarrow \text{arginine} \rightarrow \text{ornithine} + \text{urea.}$$

Evidence has been obtained by Srb & Horowitz (79) for the operation of a similar reaction sequence in *Neurospora*. Information is accumulating that at least a portion of the above cycle may be operative in higher plants. Thus, Miettinen & Virtanen (80) have reported that alder roots contain large amounts of ornithine, citrulline, and arginine, but little or no urea. They find that arginase, the enzyme which splits arginine to ornithine plus urea, is, however, absent. Further evidence for the occurrence of at least a modified urea cycle in plants comes from the demonstration by Walker (81) that arginosuccinic acid is formed by acetone-dried *Chlorella* cells in the presence of malate or fumarate. Similar observations were made by Davison & Elliott (82) with extracts of dried peas or of lupine seeds. The purified enzyme (from peas) requires both arginine and fumarate as substrates. Malate is inactive. The specificity of the *Chlorella* reaction for fumarate was reported later by Walker & Myers (83), who isolated a crude enzyme from *Chlorella* cells. They further observed that *Chlorella* contains an arginine desimidase which catalyzes the reaction:

$$\text{arginine} \rightarrow \text{citrulline} + NH_3,$$

but that citrulline formation from arginosuccinate could not be observed. In another communication (84) the reaction of canavanine, an amino acid related to arginine which occurs in high concentration in jack beans, with fumarate, to form canavanosuccinate was described.

The significance of these observations may be twofold. Walker (81) has suggested that arginosuccinate may be of importance in pyrimidine synthesis.

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Alternatively, arginosuccinate may be an intermediate in urea assimilation in cells which lack urease. *Chlorella* cells grown on urea, for example, contain no detectable urease (81) but accumulate arginine. Walker suggests, therefore, that urea may be metabolized by condensation with ornithine to form arginine. This he suggests might be followed by transformation of arginine to citrulline or to arginosuccinate and further metabolism. In this connection, the recent report of Williams & Sharma (85) that urease is absent from *Citrullus* species emphasizes our need for further information concerning urea metabolism in plants.

Metabolism of specific amino acids: lysine.—The conversion of radioactive lysine to pipecolic acid in plants has been reported by Lowy (86) and by Grobbelaar & Steward (87). The high specific activity of the pipecolic acid isolated indicates that pipecolic acid represents one of the earliest products of lysine breakdown. Similar results were obtained by Rothstein & Miller (88) in mammalian tissues. Recent findings by these investigators (89) and by Schweet, Holden & Lowy (90) support the view that the first step in lysine metabolism is the loss of the α-amino group and that this is followed by cyclization to pipecolic acid. There are also (91) strong indications that α-aminoadipate, glutarate, and α-ketoglutarate are products of the further breakdown of lysine in mammalian tissues. The steps (91) in lysine metabolism suggested by Rothstein and Miller are summarized in Figure 2.

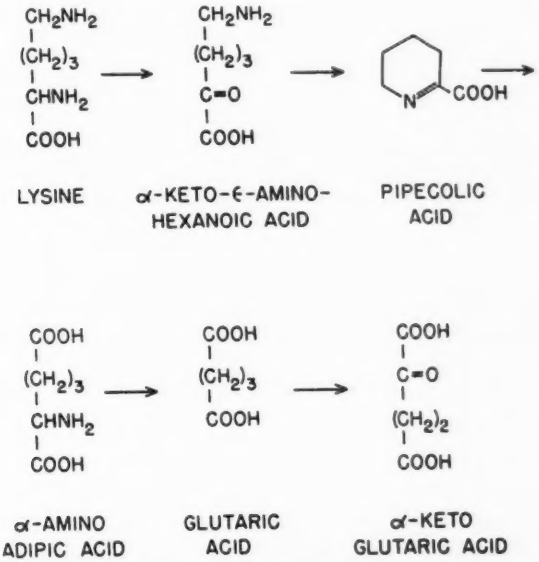

FIG. 2. Suggested pathway of lysine metabolism in mammalian tissues.

In view of the marked similarity of the lysine-pipecolic acid transformation in higher plants and animals, it would not be surprising if similar metabolic sequences were to be found in both kinds of organisms. It is noteworthy, therefore, that α-aminoadipate has been found to occur in a number of plants [Berg *et al.* (92); Windsor (93)].

Metabolism of specific amino acids: glycine.—Racusen (94) has examined the metabolism of glycine-2-C^{14} in soybean leaves. Large amounts of radioactivity were incorporated into starch and protein. Of the protein amino acids, serine contained the most activity, followed by glycine, alanine, glutamate, aspartate, leucine, and phenylalanine. Of particular interest is the extensive conversion of glycine to serine. The pathway of this conversion has been elucidated by the combined efforts of a number of investigators (95, 96, 97) who have used enzymes from mammalian tissues, and it is likely that a similar mechanism operates in plants. The mammalian conversion of glycine to serine follows the pathway outlined below:

glycine → glyoxylate → "active" formate
"active" formate + glycine → serine.

The "active" formate appears to be N-formyl-tetrahydrofolic acid (97). This reaction sequence does not appear to be appreciably reversible. As suggested earlier in this review, the transformation of serine to glycine may proceed by the following pathway:

serine → ethanolamine → glycolate
glycolate → glyoxylate → glycine

The rapid conversion of glycolate to glyoxylate was demonstrated several years ago by Tolbert, Clagett & Burris (98). In addition, Tolbert & Cohan (99) have demonstrated the formation of glycine from glycolate, apparently through the intermediate formation of glyoxylate. A further pathway of glycine metabolism in plants, the transformation of glycine to choline, apparently via serine and ethanolamine, has been reported by Bregoff (100).

Amide and Peptide Bond Synthesis

The conversion of amino acids into more complex substances (amides, peptides, and proteins) has received an increasing amount of attention during the past two years. The reverse process, the enzymatic breakdown of glutamine, asparagine, and simple peptides into their constituent amino acids has long been known. However, the equilibrium for the hydrolytic breakdown of both amides and peptides is so far in the direction of the free amino acids (7) that the biosynthesis of these substances by a simple reversal of hydrolysis is practically impossible. It is now known that the *in vivo* synthesis of amides and peptides is achieved by linkage of the reaction to a second energy-yielding reaction. The energy source has been shown in every case examined to be satisfied by ATP.

Glutamine synthesis.—The first demonstration of glutamine synthesis in a cell-free plant system was achieved by Elliott (101) who used homogenates of *Lupinus* seedlings. Elliott likewise found that the synthesis requires both ATP

and magnesium ions. The dependence of glutamine synthesis on ATP and Mg^{++} was confirmed by Webster (102) with extracts of bean seedling acetone powders as the enzyme. The stoichiometry of the reaction as determined by both investigators is

$$\text{glutamate} + NH_3 + ATP \rightleftarrows \text{glutamine} + ADP + P_i.$$

The enzyme is mitochondrial (102). The location of such a synthetic enzyme on the mitochondria would appear to be most appropriate since it is then favorably placed in relation to the ATP-generating system of the cell (103).

Although enzymes catalyzing glutamine synthesis have also been found in mammalian tissues (104) and bacteria (105), it is only the plant enzyme which has as yet been purified to the extent necessary for further critical study of the synthetic reaction. Elliott (106) purified the glutamine synthetase from dried green peas some 2000-fold over the original extract. The protein was not homogeneous, exhibiting three peaks electrophoretically and two peaks ultracentrifugally. By modifying and extending the procedure of Elliott (106), Varner & Webster (107) were able to purify the glutamine synthetase from green peas some 4100-fold. This enzyme, which approaches homogeneity, exhibits three activities of interest. These are: (*a*) glutamine synthesis, (*b*) glutamyl transfer, and (*c*) arsenate-activated glutamine hydrolysis. The glutamine synthesis reaction has now been rather completely characterized (106, 107). In addition to the substrates (glutamate, ammonia, and ATP) the synthetic reaction requires magnesium, but not potassium ions. The report of Dénes (108) that cobalt greatly enhances glutamine synthesis could not be confirmed by Varner & Webster (107). Synthesis is strongly inhibited by *p*-chloromercuribenzoate, and the inhibition is completely eliminated by β-mercaptoethanol. Iodoacetate, however, fails to inhibit the enzyme. The reversibility of the synthetic reaction has been demonstrated both by Levintow & Meister (109) and by Varner & Webster (107). The equilibrium constant calculated for the forward reaction is 1.2×10^3 at pH 7.0 (Levintow and Meister) and 1.7×10^3 at pH 7.4 (Varner & Webster). From this equilibrium constant, Varner & Webster (107) (using the accepted value of $-10{,}500$ cal. for the standard free energy of hydrolysis of ATP) calculated the free energy of formation of the amide bond of glutamine to be about 6000 cal. A completely different interpretation of these results has been offered by Levintow & Meister (109). Although no direct determination of the free energy of hydrolysis of glutamine is available, they assume that it is essentially the same as that of many peptide bonds, namely, about 3500 cal. From this they calculate that the free energy of hydrolysis of ATP is actually only about -8000 cal. Interestingly enough, support for this suggestion comes from recent studies on the E'_o of the DPN^+/DPNH system. Burton & Wilson (110) have calculated a revised value for the E'_o which is 0.320 v. at pH 7. rather than the accepted value of 0.28 v. (111). As Anfinsen & Kielley (112) have pointed out, the new value for the DPN^+/DPNH system requires a correction in the value calculated by Oesper (113) for the free energy of hydrolysis of ATP. The new value calculated on this basis

is 8640 cal., not greatly different from the revised value suggested by Levintow and Meister. In view of the importance of both ATP and glutamine in cellular physiology, it will be of interest to study further the free energy of hydrolysis of the two compounds.

Glutamyl transfer.—The catalysis of the glutamyl transfer reaction by highly purified glutamine synthetase (106, 107, 109) is noteworthy because it appears to explain the puzzling significance of this reaction. Glutamyl transferase was found in bacteria by Grossowicz *et al.* (114) and in plants by Stumpf & Loomis (115) and catalyzes the following reactions:[2]

$$\text{Glu-NH}_2 + \text{N}^{15}\text{H}_3 \rightleftarrows \text{Glu-N}^{15}\text{H}_2 + \text{NH}_3$$
$$\text{Glu-NH}_2 + \text{NH}_2\text{OH} \rightleftarrows \text{Glu-NHOH} + \text{NH}_3.$$

The importance of these reactions in plant metabolism, however, remained obscure until Elliott (106) found that the ratio of synthetic to transfer activity remained constant throughout the purification of the glutamine synthesis enzyme. Varner & Webster (107) also obtained a constant ratio of the two activities during preparation of the 4100-fold purified enzyme. These findings have led to the suggestion (107, 109) that glutamyl transferase is merely a manifestation of glutamine synthesis activity. This suggestion is in line with the transfer activities of other synthetic enzymes (116, 117) and is supported by the similarity of properties of the two activities (107). The view that glutamyl transfer is simply a part of the glutamine synthesis system eliminates any necessity for postulating a physiological function for glutamyl transfer in the cell. The present author is inclined toward this view and sees no good evidence at present to contradict it.

The mechanism of the glutamyl transfer reaction has only recently received attention. Levintow & Meister (109) have suggested that "the transferase reaction may be visualized as reversal of synthesis, followed by reaction of the ATP and glutamate formed with hydroxylamine, regeneration of ADP and phosphate, and repetition of the cycle." This interpretation is, however, inconsistent with several facts. First, the extremely low concentration of ATP formed as a result of the reverse reaction would not permit the forward reaction to proceed at a rate at all comparable with that actually observed (107) in the transfer reaction. The suggestion of Levintow and Meister would also lead to the surprising conclusion that the enhancement of transfer activity in the presence of arsenate instead of phosphate would be attributable to the formation of the ADP-arsenate analogue of ATP and that this compound is more effective than ATP in the synthesis reaction. Varner & Webster, however, (107) have failed to find any evidence for the formation of an ADP-arsenate-like compound during the reaction. Finally, these workers (107) have shown that essentially no equilibration occurs between radioactive glutamate and glutamine during the glutamyl transfer reaction. It is, therefore, necessary to conclude that glutamate remains enzyme-bound during the course of the transfer reaction. The following mechanism is consistent with these facts:

$$\text{E} + \text{Glu-NH}_2 + \text{P}_i + \text{ADP} \rightleftarrows \text{E(ATP)-Glu} + \text{NH}_3$$
$$\text{E(ATP)-Glu} + \text{NH}_2\text{OH} \rightleftarrows \text{E} + \text{Glu-NHOH} + \text{P}_i + \text{ADP}$$

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or

$$E(ATP)\text{-}Glu + N^{15}H_3 \rightleftarrows E + Glu\text{-}N^{15}H_2 + P_i + ADP.$$

The important point is the binding of glutamate to the enzyme (E), which conserves the energy of the amide bond. This binding depends on the presence of adenylphosphate. The effectiveness of arsenate in the reaction may be attributable to the greater ease of cleavage of the arsenate bond in the transient intermediate as compared to the phosphate bond.

An arsenate-activated hydrolysis of glutamine is also catalyzed by glutamine synthetase (107, 109). The reaction has an absolute requirement for ADP, arsenate, and either magnesium or manganese ions (107, 109). It is thus very similar to the transfer reaction. Such a hydrolysis proceeds only extremely slowly in the presence of phosphate instead of arsenate. It might be envisioned that the active intermediate, E(ADP-AS)-Glu,[2] is unstable in the absence of an amine acceptor to the extent that it spontaneously decomposes to give free glutamate.

Mechanism of synthesis.—The mechanism of the glutamine synthesis reaction has been partially elucidated. Attempts by several investigators to detect the formation of a glutamyl phosphate intermediate have been consistently negative (106, 107, 118). Likewise, the participation of a CoA intermediate has been rendered unlikely by the investigations of Elliott (106) and of Webster (102). It now appears that enzyme-bound intermediates participate in the synthesis (107). Although early results (119) indicated the mechanism of glutamine synthesis to be similar to that found by Webster & Varner (116) for peptide synthesis, later experiments (107) with the highly purified enzyme have indicated that all components of the reaction system remain bound to the enzyme until the addition of ammonia triggers release of all the products of the reaction simultaneously. The evidence indicates an overall reaction mechanism of the following type:

$$Glu + ATP + E \rightleftarrows E(ATP)\text{-}Glu$$
$$E(ATP)\text{-}Glu + NH_3 \rightleftarrows E + Glu\text{-}NH_2 + ADP + P_i.$$

The suggested mechanism is not only consistent with all of the information on exchange reactions, but makes understandable also the absolute requirement of both ADP and P_i for the transfer reaction. It would appear that elucidation of the exact mechanism by which ATP participates in glutamine synthesis must await new investigational techniques. There is little doubt, however, that a glutamyl-enzyme entity is the active intermediate in synthesis.

Asparagine synthesis.—Although asparagine enjoys an importance in plant metabolism which parallels that of glutamine, information on its biosynthesis has lagged far behind that regarding glutamine. The presence of asparaginase in plants seems fairly certain. That asparagine synthesis does not occur by the mediation of asparaginase is evident from thermodynamic studies on asparagine hydrolysis (7) which show that equilibrium lies over 99 per cent toward hydrolysis. This conclusion is supported by the recent studies of Tannenbaum, Garnjobst & Tatum (120) on an asparagine-requiring mutant of *Neurospora*. The genetically determined inability of this mutant to synthesize asparagine

is apparently a result of the failure of a single biochemical step in the synthesis. That the failure is not attributable to a lack of asparaginase is shown by the fact that this enzyme is equally active in mutant and wild type *Neurospora*. The enzyme which mediates the synthesis is, therefore, concluded to be distinct from asparaginase.

There are several obvious possible pathways for asparagine synthesis in plants: (*a*) direct amidation of aspartate in the presence of ATP, in analogy to glutamine synthesis; (*b*) transamidation between aspartate and an amide (possibly glutamine); (*c*) carboxylation of β-alanylamide; (*d*) amination of α-ketosuccinamate. Of these, reactions (*b*) and (*c*) have been fairly well eliminated by experiments of Webster & Varner (121). On the other hand, Meister & Fraser (122) have found that the amination of α-ketosuccinamate to asparagine is catalyzed by an enzyme from mammalian tissues. Unfortunately, the synthesis of the amide bond of α-ketosuccinamate has not been demonstrated, and it is, therefore, still questionable whether or not this reaction participates in asparagine synthesis within the cell. Webster & Varner (121) have, however, demonstrated that extracts of lupine seedlings or of wheat germ catalyze the synthesis of radioactive asparagine in the presence of ammonia, ATP, magnesium ions, and radioactive aspartate. If ammonia is replaced by hydroxylamine, aspartyl hydroxamate is formed. The system also carries out an aspartyl transfer reaction similar to glutamyl transfer. Although it has not been proven as yet that this reaction constitutes the only pathway of asparagine synthesis in plants, this possibility certainly must be seriously considered and subjected to further experiment.

Peptide bond synthesis.—The formation of peptide bonds by a direct condensation of amino acids has only recently been investigated in plants. It was first observed [Webster (123)] that incubation of bean hypocotyls with glutamate, cysteine, and glycine resulted in a marked increase in the glutathione level of the tissues. If radioactive glycine was used, radioactive glutathione was formed. The formation of glutathione could also be demonstrated in homogenates of bean hypocotyls (123) as well as in a variety of other tissues. With soluble enzyme systems, it was possible to demonstrate a requirement of the synthesis for ATP and for both magnesium and potassium ions. Kinetic studies indicated that the formation of glutathione probably proceeded in two steps: the initial formation of a dipeptide, glutamylcysteine, followed by the addition of glycine to this dipeptide to form glutathione. Evidence that this is so was obtained when it was shown [Webster, (124)] that enzyme preparations which catalyze the synthesis of glutathione also catalyze the synthesis of glutamylcysteine. Webster & Varner (125, 126) have now isolated two separate enzymes from wheat germ which carry out the suggested steps in glutathione synthesis. The first enzyme (125) specifically catalyzes glutamylcysteine formation by the reaction:

$$\text{Glutamate} + \text{cysteine} + \text{ATP} \rightleftarrows \text{Glutamylcysteine} + \text{ADP} + P_i.$$

The second enzyme (126) forms glutathione from glutamylcysteine:

$$\text{Glutamylcysteine} + \text{glycine} + \text{ATP} \rightleftarrows \text{Glutathione} + \text{ADP} + P_i$$

The properties of the two enzymes are remarkably similar. Both require magnesium and potassium ions for activity. Their general enzymatic characteristics (pH optimum, substrate relations, dependence on sulfhydryl groups) are likewise almost identical.

Mechanism of peptide bond synthesis.—In an attempt to determine whether or not a γ-glutamyl phosphate is an intermediate in glutamylcysteine synthesis, Webster & Varner (125) measured the exchange of phosphate residues between orthophosphate-P^{32} and ATP as catalyzed by the purified synthetic enzyme. It was found that such exchange did not occur except in the presence of glutamate. Conversely, the exchange of ADP^{32} into ATP proceeded readily and was slightly decreased by the presence of glutamate. The enzyme also catalyzed the exchange of cysteine-S^{35} with glutamylcysteine. On the basis of these facts, Webster & Varner (125) have suggested the following mechanism:

$$E + ATP \rightleftarrows E\text{-}P + ADP$$
$$E\text{-}P + Glu \rightleftarrows E\text{-}Glu + P_i$$
$$E\text{-}Glu + Cys^* \rightleftarrows Glu\text{-}Cys + E.$$

Similar proposals have been made by both Snoke (127) and Webster & Varner (126) in connection with glutathione synthesis by enzymes from yeast and wheat germ, and by Maas (128) in a study of the enzyme from *Escherichia coli* which catalyzes pantothenate synthesis. The proposed mechanisms agree excellently in each case with the exchange results and make understandable otherwise puzzling phosphate exchange phenomena. They interpret also the lack of evidence for formation of substrate-phosphate or substrate-cofactor intermediates in the reactions. The proposed mechanisms are undoubtedly only first approximations to the actual reaction sequences which may well be more complex. With this qualification, however, such proposals are valuable as working hypotheses, and suggest further experiments which can eventually lead to understanding of the detailed mechanism of the synthetic reactions.

The relationship of the peptide bond syntheses discussed above to the larger and more important problem of protein synthesis is not yet clear. Since glutathione is important as a cofactor in carbohydrate metabolism, it is entirely possible that its synthesis might be unique and that the formation of its constituent peptide bonds might bear no relation to the formation of the peptide bonds in proteins. In this connection, it should be pointed out that no relationship between glutathione metabolism and protein synthesis has ever been demonstrated. Although Hanes, Hird & Isherwood (129) found that a variety of γ-glutamyl peptides are formed from glutathione by transfer reactions, no evidence of the importance of such transpeptidations to protein synthesis is yet available. In fact, Hendler & Greenberg (130, 131) found with liver preparations that the glycine of γ-glutamyl glycine is incorporated much less rapidly into protein than is free glycine. Despite this lack of evidence in favor of a direct participation of glutathione in protein formation, evidence is beginning to accumulate that the mode of formation of its peptide bonds may be a general model for the formation of peptide linkages in protein. Thus, Webster (132) found that the incorporation of radioactive amino acids into

plant mitochondrial protein is dependent upon ATP and upon both magnesium and potassium ions. The optimal conditions for peptide bond synthesis in glutathione are likewise optimal for amino acid incorporation into protein. It would thus seem that studies on such simple systems as glutathione may be of importance in probing the more complex systems which participate in protein synthesis.

Protein Synthesis

Probably no process that occurs in living cells (except perhaps photosynthesis) has been the subject of as much speculation as has the synthesis of protein. In spite of the generous amount of discussion devoted to the subject, the simple fact remains that the manner in which protein is formed is still quite obscure. The mechanisms that have been proposed to account for protein synthesis may be roughly divided into four groups: (*a*) Reversal of protein hydrolysis; (*b*) Synthesis by condensation of amino acids on a template; (*c*) Synthesis by transpeptidation between preformed peptides; (*d*) Synthesis by the stepwise condensation of small peptides. There is, unhappily, both evidence for and evidence against each theory of protein synthesis thus far proposed. It is possible, in fact, that different proteins (virus proteins, adaptive enzymes, reserve proteins) may be formed by entirely different processes. It seems to the present reviewer that most of the recent speculation on protein synthesis in plants has contributed little to the advance of our knowledge of this process. Rather than extending such discussion (particularly in relation to the above four theories), the present review will be confined to comment on recent experimental results concerning protein formation.

Protein synthesis in ripening seeds.—Danielsson and co-workers (133 to 136) have shown that ripening peas form two easily isolated globulins, vicilin and legumilin. The proteins have been characterized by Danielsson and shown to be electrophoretically homogeneous. The globulins are somewhat different in amino acid composition and are likewise markedly different from the amino acid composition of the heterogeneous albumin fraction. The globulins are apparently true reserve proteins and provide the amino acids for formation of cellular protein during germination. The globulins are formed most rapidly just after the ripening begins and the synthesis of protein is inversely related to the level of free amino nitrogen (134). Danielsson has further shown that protein formation proceeds even in detached seeds. This direct and easily measurable formation of specific proteins makes the pea system one of the most attractive yet proposed for the direct study of protein synthesis.

Protein turnover in detached leaves.—The leaf is another excellent organ for the study of protein metabolism. The growing leaf increases many times in protein content during growth. The virus-infected leaf likewise provides an opportunity for the study of the synthesis of a new and apparently quite unique protein. The mature leaf can be used for studies on protein turnover, while detached leaves exhibit a striking decrease in protein content. The cause of protein loss in detached leaves has evoked numerous experiments and speculations

[for an excellent review of the literature up to 1950, see Bonner (137)]. Investigation of the problem has fortunately been renewed. Both Racusen & Aronoff (46) and Chibnall & Wiltshire (138) report that protein synthesis may be proceeding during the rapid decline in protein level in detached leaves. Chibnall (139) has shown that protein breakdown is inhibited when adventitious roots are induced on the petiole. He concludes that roots produce some factor or factors necessary for the maintenance of normal protein levels. This phenomenon suggests an interdependence between organs which may be of potential importance to our understanding of protein metabolism. If it be that one organ of the plant supplies factors regulating protein metabolism in another organ, then the possibility of chemical control of protein metabolism in individual organs is presented. The most obvious possibility in this root-leaf relation is that roots, which are known to form all of their amino acids, supply the leaf with amino acids which leaves cannot make. The possibility that leaves cannot make all of their amino acids has been examined both by Racusen & Aronoff (46) with $C^{14}O_2$ and by Rogers (41) with C^{14}-sucrose and C^{14}-acetate. Racusen & Aronoff report that at least young leaves in the light can apparently incorporate $C^{14}O_2$ into most, if not all, of their amino acids. Rogers has found that leaves incorporate radioactive carbon from sucrose into every one of the amino acids ordinarily found in protein. In some cases, the rates of formation are, however, quite slow. It is evident from these results that the dependence of leaf protein levels upon the presence of roots is not a simple dependence upon a source of some "essential" amino acid not made by the leaf. It is possible, of course, that the rate of synthesis of some amino acid is so slow in leaves that they are dependent upon roots for a supply that is adequate to maintain protein levels. It would be of interest to determine whether or not culture of excised leaves in a medium containing a complete spectrum of amino acids inhibits protein breakdown. It seems increasingly possible, however, that the effect of roots on protein level in leaves may be attributed to some factor other than an amino acid which is supplied by roots. Perhaps relevant to this question are the extensive studies of Bonner (140) on the growth of excised leaves. He found that the growth of leaf tissues was markedly enhanced by the presence of adenine in the sucrose nutrient medium. Excised leaf tissues also responded to certain amino acids. Unfortunately, information is not available as to whether adenine and amino acids also inhibit protein breakdown in excised leaves. It would seem, however, that feeding experiments with excised leaves may offer the best opportunity at present for elucidating the contribution of roots to the protein level of leaves.

Another approach to the question of what roots contribute to protein level in leaves would be to study the nitrogenous substances that move upward from root to leaf. Information on the translocation of nitrogenous substances is, however, very limited. Bollard (141) has examined the nitrogenous products of apple tree sap by paper chromatography. Approximately 90 per cent of the nitrogen translocated was in the form of glutamate, as-

partate, glutamine, and asparagine. Lesser amounts of serine, threonine, arginine, methionine, valine, leucine, alanine, tyrosine, γ-aminobutyrate, and unidentified peptides were present. Mothes and co-workers (142, 143) have examined the saps of several plants. The major constituents appear to vary from plant to plant. They find quantities of allantoin and allantoic acid in maple trees, while nitrogen appeared to be transported chiefly as asparagine in the elm, and as citrulline in the alder. Definite quantities of other amino acids, such as proline, glutamate, and aspartate were also present, however. It is noteworthy that Wolffgang & Mothes (143) likewise indicate the translocation of a peptide in their work. This peptide is composed of glutamate and an unidentified, ninhydrin-positive substance. The peptide also occurs in relatively high concentration in the sap of sunflowers. Our lack of information on the occurrence of peptides in living organisms makes the further identification of this peptide of definite interest. The results obtained by paper chromatography thus far show that a wide variety of nitrogenous substances are translocated in plants. Undoubtedly, many substances remain to be identified, any of which may prove to influence protein levels in leaves. A lucid discussion of root-shoot relations (particularly in nitrogen metabolism) has recently been presented by Mothes (144).

Amino acid incorporation into protein.—While the studies discussed above increase our understanding of the physiological aspects of protein metabolism, they do not promise to elucidate the intimate chemical pathways involved in protein synthesis. More direct biochemical approaches seem to offer greater chances of at least limited advancement. When a plant tissue is incubated in the presence of a radioactive amino acid, the cells rapidly accumulate the amino acid (145). The accumulation process is dependent upon respiration and thus resembles the accumulation of other substances (ions, sugars, etc.) by plant cells. The fate of the amino acid inside of the cell varies somewhat according to the amino acid and the experimental conditions. Numerous radioactive amino acids have now been investigated in the author's laboratory (145), and in every case (glutamate, aspartate, glycine, cysteine, histidine, serine, and leucine) definite and easily measurable amounts of amino acid were incorporated into protein. That the incorporated amino acid is bound through peptide linkages is indicated by several facts: the incorporated radioactivity is stable to boiling trichloracetic acid, to solution in dilute alkali, and to reducing or oxidizing agents (145). When carboxyl-labeled amino acid is incorporated, no radioactivity is liberated on treatment of the protein with ninhydrin. Hydrolysis of the protein, however, yields a product from which the radioactivity can be completely liberated as CO_2 by ninhydrin treatment. Partial hydrolysis of the protein with trypsin results in only a small liberation of activity. It seems quite likely, therefore, that the incorporated amino acids are bound through peptide linkages to the protein. Rigorous proof of this, however, must await the isolation of radioactive peptides from partial hydrolyzates, and the liberation of the incorporated amino acid by degradation of the peptide. This procedure has been

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followed in only one case (146). The results in this case show that incorporation is through a peptide bond. Further evidence that incorporation occurs through the formation of peptide bonds is found in recent work [Webster (132)] on incorporation of glutamate, aspartate, and glycine by plant mitochondria. That incorporation is dependent upon an ATP supply was shown by its enhancement by Krebs cycle intermediates plus adenylate, and its inhibition by cyanide, azide, and dinitrophenol. ATP is able specifically to overcome the cyanide inhibition of incorporation, thus indicating directly the role of ATP in the incorporation process. The necessity of an energy source for incorporation makes it unlikely that incorporation occurs by simple adsorption on the protein.

The pattern of incorporation varies greatly among the different particulate fractions of the cell. The distribution of such incorporation is summarized in Table I. It is evident that properly fortified homogenates of a

TABLE I

INCORPORATION OF RADIOACTIVE GLUTAMIC ACID BY INTACT BEAN HYPOCOTYLS, BY HOMOGENATES OF THESE HYPOCOTYLS, AND BY ISOLATED CELLULAR PARTICLES

Glutamic acid-2-C^{14} incorporated (μM/hr./gm. protein)

Cellular fraction	Intact tissues	Homogenate	Isolated particles
Nuclei	0.15	0.18	0.11
Mitochondria	0.43	0.55	0.38
Microsomes	1.18	1.83	0.09
Soluble	0.31	0.59	0.25

tissue show a distribution of radioactivity very similar to that of the intact tissue itself. Greatest incorporation occurs into the small particle (microsomal) fraction and least into the nuclear fraction. When isolated microsomes are incubated with amino acids, however, no significant incorporation occurs, indicating a dependence of incorporation upon other cellular fractions. In particular, it has been found (147) that a mixture of mitochondria plus microsomes carry out rapid incorporation in the presence of ATP and citrate.

The lack of amino acid incorporation by the nuclei is likewise of interest. Thoughts on protein synthesis have generally revolved around some relation between nucleic acid and protein synthesis. In this connection, the nucleus has often been considered to be involved directly in the formation of protein. The incorporation studies suggest that there may be no direct connection, however, since synthesis would of necessity involve relatively great amounts of incorporation in nuclei. There are two places in the cell, however, where nucleic acids are concentrated, DNA^2 in the nuclei and also RNA^2 in the microsomes. The high incorporation ability of microsomes suggests that it

may be here that a connection between nucleic acid and protein synthesis is found. Relative to this, recent experiments by the author (147) indicate that treatment of microsomes from pea seedlings with crystalline ribonuclease completely stops their ability to incorporate amino acids. Furthermore, Gale & Folkes (148) have now shown that incorporation of amino acids in bacterial cell breis is greatly enhanced if nucleic acid or a mixture of purines and pyrimidines is present. The relation between nucleic acids and protein synthesis is, therefore, obscure but apparently real.

Of what importance are incorporation studies to studies of protein synthesis? Probably their greatest value thus far has been the demonstration of their marked similarity in properties to the synthesis of simple peptides. This has allowed the knowledge gained in these simple systems to be transferred to a more complex phenomenon. Undoubtedly, at least a portion of the incorporation hitherto studied in plants, animals, and bacteria occurs by some exchange process catalyzed by the protein-forming system. A part of it, however, may occur by actual protein synthesis. Cell-free incorporation systems would seem to offer an opportunity for study of protein formation, particularly if such systems can be caused to carry out a net synthesis of proteins. That it may indeed be possible to obtain protein formation in cell-free systems has been indicated by the experiments of Gale & Folkes (148) with broken cell preparations of bacteria. They have shown that incorporation of a single radioactive amino acid into protein is greatly enhanced in the presence of a complete mixture of 17 other unlabeled amino acids. Furthermore, this enhanced incorporation is even further increased in the presence of a mixture of purines and pyrimidines. With this enriched system, it has been established that the activity of several enzymes in the bacterial brei increases markedly during incubation, and that this increase is dependent upon the presence of the mixture of amino acids. Gale and Folkes suggest this may result from the actual synthesis of the individual enzymes. Although this must still be proven, it would appear that the study of amino acid incorporation in cell-free systems may usher in a new era of our knowledge of protein synthesis.

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FUNCTIONAL ASPECTS IN MINERAL NUTRITION OF GREEN PLANTS[1]

By A. Pirson

Botanical Institute, University of Marburg (Lahn), Germany

Introduction

Reviewers in the field of mineral nutrition of plants have always been faced with a particular difficulty. Mineral nutrition is an area of research which is hard to delimit, since it branches far into the various spheres of plant physiology. The more a particular problem is delineated and a solution to it sought, the more it appears to belong to other fields, such as physiology of metabolism, physical cell physiology, or enzymological biochemistry. In addition, applied research is constantly turning up data which, although intended for practical purposes, cannot be ignored by the researcher who is interested in theory. Thus, isolated results sometimes uncorrelated and often merely confirming others multiply from year to year to an uncountable number. In a report such as this it would be pointless to try to deal with the whole literature; certain limits must necessarily be set, and in setting such limits a certain subjectivity is naturally unavoidable. In this report the author has deliberately emphasized certain functional-dynamic aspects in the work on mineral nutrition, which offer hope for future progress in a causal analysis of mineral effects. In some fields there have been, in spite of a number of publications, no important new findings which would appear useful in this regard. These will therefore be referred to here only slightly, if at all. Such a markedly personal evaluation of research methods and results is, whether affirmative or negative, admittedly open to criticism. In any case the one-sidedness of the treatment here may be compensated for by the fact that the subject will soon be reviewed from another standpoint.

All questions of salt uptake have been omitted, since these are being dealt with in another article in this volume. Of nitrogen metabolism only partial problems in the purely mineral field have been touched, in order to establish a connection with another chapter of this volume. Purely enzymological data need only to be mentioned in passing, since there was recently an impressive and detailed report here on this subject (173). The results obtained on algae have received relatively strong emphasis; therefore, the comprehensive description of mineral requirements in phytoplankton by Ketchum (148), and the treatment of algal physiology by Myers (187) and Fogg (89) deserve to be mentioned.

Of the various synoptic presentations of the whole field which have appeared in recent years, the concise book of Baumeister (27) and the review (Wisconsin Symposium) edited by Truog (276) may be mentioned. There

[1] The survey of the literature pertaining to this review was completed in September, 1954.

have also been presentations, with extensive bibliographies, of the problems of micronutrients (114, 116, 289) and the technique of sand and water cultures of higher plants (115, 248).

General Considerations

Essentiality of elements.—Many problems of mineral nutrition are contained in the three criteria of essentiality which Arnon [(7, 8), cf. (15)] has formulated. They have often been used in discussions by specialists, and may be mentioned here once more in order to avoid unclarity and misunderstandings. An element may be considered essential if (*a*) the complete process of vegetative or reproductive development of the plant is prevented by the absence of the element; (*b*) a deficiency symptom is specific for the element, and can be corrected only by adding the element; (*c*) the element plays a direct part in the nutrition of the plant, and does not exert its favorable influence through some factor lying outside the organism.

The first criterion includes the requirement that all biochemical and especially enzymological evidence must be critically tested for validity *in vivo;* i.e., the living plant itself must always be asked. This question is still acute, especially in the evaluation of double or multiple activation of enzymes *in vitro*, i.e., of enzymes with low ion specificity, or, even more generally, in the case of the physiological effects of those ions for which the biochemist has not yet found a satisfactory place as constituents of biogenic molecules. In a study of the possible functions of metal ions in biological systems Williams (303) has given examples to show that ion series with graduated activation effect in nonenzymatic catalyses are relatively fixed according to the degree of their tendency to complex formation, and not so much according to the type of the particular ligand. But if such a catalysis series is compared with that of an equivalent enzymatic, i.e., protein-bound reaction *in vitro*, then no agreement of results can be noted any more. Changing over to a living system, to the extent that this would be at all possible, would certainly bring a new alteration of the apparent activating effect of the ions in question. It is doubtless very interesting to trace such activations, inactivations, antagonisms, and synergisms, together with their causes. But here it is not a question of effects which are related to the question of the essentiality of an element. The problem of such ion effects was made especially clear not long ago, when chloride ions were claimed to be activators ("coenzymes") in the Hill reaction of isolated chloroplasts, and, from this consideration alone, nutritional requirements were deduced [(291), cf. (8, 17)]. Also concerning the question of the replaceability of elements the biochemical analysis must be related to, or tested by, biological evidence. There are no certain grounds yet for believing that one of the known macro- or micro-nutrients can be replaced by another; thus there should be no reason to criticize the second of the above criteria. If isotopes, aside from possible disturbances by radiation effects, do not constitute, for purely physical reasons, absolutely identical substituents, then there is even more reason to

assume that disturbing factors arise from substitution by other elements. These apparently make themselves felt the more strongly, the more complicated is the cytological and histological structure of the organism. In regard to the ion pairs Ca^{++}/Sr^{++} and K^{+}/Rb^{+}, substitution in microorganisms with only low morphological differentiation appears to come fairly close to biological replacement (210, 215, 288).

Arnon's criteria do not explicitly include cases in which the requirement of an element is limited to a particular nutritional form. However, evidence seems to show that this possibility can, in most cases, be practically ruled out. In the first phase of the investigation of a micronutrient such a special limitation has often been assumed, e.g., with manganese or molybdenum (nitrate reduction, nitrogen fixation). But further research has revealed a more complicated effect of these elements, since they have a direct, activating effect either on several systems or on a central point in metabolism, the influencing of which must express itself in a number of secondary symptoms. According to the present state of knowledge, a connection of manganese with the autotrophic form of nutrition (32, 211) seems to be proved in algae of the *Chlorella* type; this may be true also of the limitation of calcium requirement in certain bacteria to a supply with certain nitrogen sources (105).

The search for micronutrients important to life has been stimulated by the unexpected demonstration of a vanadium requirement in *Scenedesmus* (16). Molybdenum (14, 167, 288) and even copper (288) are now proved to be micronutrients in green algae. These new successes are attributable entirely to marked improvements in experimental technique. Numerous methods of purification (water, chemicals, containers), some of them designed especially for single elements, have been worked out by Hewitt (115, 119) from his own experience, and have given indispensable aid to future research. Further special purification techniques for microbiological media have also been developed (67). At present we have not yet progressed far from the oft-mentioned but purely formally conceived "nutrient line" of Frey-Wyssling (92); but further surprises here are quite possible. The question has even been raised whether, in the important regulation center of the cell in extreme cases, perhaps a single molecule of a vital element is active (192).

Analysis of the function of essential elements.—The plant physiologist should also be impressed by the possibilities offered by the refined methods of visual diagnosis in the recognition and specification of deficiency symptoms [cf., most recently, Wallace (290)]. This has mainly a practical significance, as is the case in testing the uptake of the mineral components in leaves by means of foliar analysis; thus it may serve only as a first stimulus to physiological work. The analysis of the main organic constituents in deficient plants has not yet succeeded in going beyond the symptom stage, in spite of the use of elaborate apparatus. Steinberg (264) may be mentioned in this connection; he has compared numerous data from the literature on quantity and relationship of protein nitrogen to soluble nitrogen (also that of total sugar to reducing sugar, etc.). He has concluded that it is pointless to try

to find connections between individual deficiencies and special steps of physiological reactions in plants, since protein metabolism as a basic process is everywhere primarily affected in approximately the same manner. This skepticism throws light on the whole situation in higher plants, but in the opinion of the present writer, it still ignores certain analytical possibilities which may lead beyond this gross technique [cf. eg. (103, 232)].

In any case, it is desirable to replace or extend an excessively static treatment of deficiency symptoms with a dynamic one; i.e., to follow as closely as possible the progressive development of a disturbance, with analytical studies. However Eaton (75) has shown by examples of potassium deficiency, that even then the results are not completely clear; this is partly due to differences in species, which so often vary. For higher plants, especially, it appears difficult to determine the primary effects of the deficiency of mineral factors in the cell, since the complexity of the symptoms removes the basis for a causal analysis. However, there are prospects that by means of refined analytic techniques, more specific influences of mineral deficiency on activators of metabolism and particularly on energy carriers and single enzymes, can be brought under observation in various plants. McElroy & Nason (173) have recently reported extensively on numerous experiments on a preparative determination of enzyme status in deficient plants. Such efforts have been successful, especially in regard to enzymes with known or assumed metal coenzymes; but they can also fail if the deficiency condition does not selectively inactivate the enzyme through its coenzyme, but rather excludes the latter's protein component by blocking its synthesis or even by proteolysis. This latter is particularly pertinent in the adaptive formation of enzymes (284). For example, Nicholas & Nason (197) did not succeed in extracting the apoenzyme of nitrate reductase from molybdenum-deficient mycelia of *Neurospora*, to be reactivated by the addition of molybdate *in vitro*. On the other hand, normal mycelia furnish a preparation which can be inactivated by dialysis and reactivated by the addition of molybdenum. Similarly, unfavorable results have been obtained for carbonic acid anhydrase in zinc-deficient plants (307).

Another approach to the physiological effect of mineral factors is supplied by treating plants, as well as their tissues or cells, with excess doses of particular salts. This technique also grew out of the needs of applied botany, but it soon raised numerous physiological problems. The problems here consist especially of the fact that it has not yet been clarified whether excess of an element causes disturbances which represent direct and specific consequences of this particular excess within the cell, or whether they are dependent upon the exclusion of another element, in the simplest case according to the pattern of competitive inhibition. Often it is only a question of an inhibition of uptake or transport. As the most important problem, may be mentioned the question of the causes of the numerous chloroses resulting from an excess of an element. Also in the excess range lies the question whether the effects in a special biochemical locus result directly from the added element or its

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antagonist, or whether they are the result of a central, more general attack [cf. (132)]. But it is generally agreed that the excess symptoms caused by an element do not give evidence, in principle, of the part which this element plays within the cell in normal and subnormal ranges of concentration. It is striking that this view, which may almost be taken for granted, has not proven itself in regard to the ions which, for lack of biochemically precise information, have been termed "antagonists" in the colloidal system of protoplasm (27). Here the researchers apparently have no fear of identifying excess effects with normal functions. Thus, an antagonistic regulation of cytoplasmic consistency (swelling, viscosity, hydration) is often ascribed to the frequently-examined ion pair K/Ca, by analogy to model colloid systems in which an impressive demonstration of such ion effects can be observed [cf. (93)]. And it is true that excesses of potassium and calcium produce correspondingly opposed excess symptoms in living protoplasts. However, it has been shown in the root cells of *Lemna* (217) that such excess effects do not agree with the physiological disturbances which appear in cytoplasm in case of the corresponding deficiency. An antagonistic behavior is lacking here; potassium and calcium deficit have the same effect on the observable cytoplasmic properties and on respiration. Naturally, this is not to deny the occurrence of antagonistic ion effects in the range of subnormal concentrations. But today it should be kept in mind that protoplasm is not subjected to an ion effect as a simple colloid, but rather that the ions attack selectively cell organs and reaction centers (mitochondria, plasticides, surface films), in which changes are not microscopically observable. Morphological bases of changes in physiological reaction are as yet scarcely ascertainable in this delicate field.

Algae as test organisms in nutrition research.—If, in the field of mineral nutrition, only the organism itself is able to answer the decisive questions, it should be tried to simplify the situation by the choice of experimental objects. Unicellular green algae of the *Chlorella* type thus play an increasingly important part in this field of plant physiology, aside from their claim to recognition as a future crop plant (50). Some of the methodological advantages of enzymological techniques can be applied to these algae without incurring their disadvantages. Deficiency conditions of all types and degrees can be obtained, or reproduced in a short time. All metabolic changes connected with gas exchange can be determined immediately and with great accuracy, and can serve as sensitive indicators for the appearance of deficiency symptoms. The dynamic treatment of the problems can be extended by the use of a recovery technique which was suggested some time ago by the present writer (209); this technique observes the gas exchange of deficient cells, from the moment of addition of the deficient nutrient element through the following recovery phase. Several findings of a comparative examination of deficiency conditions conducted according to these principles are compiled in Table 1, which will be often referred to in the following sections. Here it may only be emphasized that mineral deficiencies can

TABLE I

METABOLIC CHARACTERISTICS (MANOMETRIC MEASUREMENTS) OF UNICELLULAR GREEN ALGAE AT SLIGHT TO MEDIUM MINERAL DEFICIENCIES. (SHORT-TERM RECOVERY EFFECTS OF PHOTOSYNTHESIS ARE INDEPENDENT OF CHLOROPHYLL FORMATION)*

Deficient Element	Photosynthesis at Light Saturation (209, 218)		Dark Respiration (Endogenous) (63, 218)		Short Time Oxidative Assimilation of Glucose (32, 63) During Deficiency	Nitrate Reduction (Assimilation) (218)
	During Deficiency	During Early Recovery (1–2 hr.)	During Deficiency	During Early Recovery		
Mn	inhibited	completely restored	normal	unaltered	normal	not specifically influenced
K	inhibited	partially restored	markedly increased	restored	lowered	inhibited (slowly reversible)
P	inhibited	partially restored	normal or slightly increased	supernormal	supernormal	similar to K-deficiency
N (219)	strongly inhibited	sudden but slight increase after NO_3^--addition	slightly increased or slightly lowered	far above normal (several hours) (49)	about normal	very low, strong increase during recovery
Mg	inhibited	unaltered	slightly increased	unaltered	about normal	not specifically influenced
Fe	inhibited	unaltered	normal or slightly increased	unaltered	about normal	not specifically influenced
S (212)	inhibited	unaltered	about normal	unaltered	(no experiments)	not specifically influenced

* Some recent data on photosynthesis and respiration during micronutrient deficiencies and recoveries (11, 14, 167) may be added to this list by the reader.

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be grouped (*a*) into disturbances which can be easily and quickly corrected, and thus show a direct functional connection between mineral factors and metabolic processes (photosynthesis, endogenous respiration, oxidative glucose assimilation, nitrate assimilation); and (*b*) into disturbances which can only be corrected very slowly, and whose effect on a metabolic process can thus be presumed to be indirect.

If, in this review, concepts based upon studies of green algae have been pushed to the fore, it is desirable to discuss critically the possibilities and limitations of their application.

One objection, which will be made especially by researchers who are accustomed to working on higher crop plants, has to do with the general validity of findings on unicellular algae. The model character of these organisms is, as far as an organism can be considered at all as a model, generally recognized in photosynthesis research. The situation in regard to mineral nutrition is more difficult, since here it must sooner be taken into consideration that species-linked differences, perhaps for phylogenetic reasons, occur in the requirement and the function of an element. Here it is also by no means certain whether species differences of a quantitative kind, such as can often be observed, are not partially due to basically different biochemical mechanisms. However, differences in biochemical mechanisms among species can be as helpful as similarities in understanding the general nature of a physiological process. Experimental comparisons of the reactions of microorganisms and those of higher plants are sometimes doomed to failure because of purely methodological difficulties. Attempts have occasionally been made to use Lemnaceae as a convenient transition species between lower and higher plants [cf. (162, 217, 262, 300, 301)]. But the success which has been achieved here cannot disguise the fact that the simple construction of these organisms does not at all mean an equally simple physiological organization. They are subject to yearly periodical physiological fluctuations (214) and aging symptoms which are hardly less complicated than those of normally-formed higher plants. Even in algae, rhythms of development and reaction are not as uncommon as might have been desired in "model plants" [cf. (175, 202, 216, 273)].

A second difficulty arises from the use of the concept of direct or primary physiological effect. If a metabolic process (e.g., photosynthesis) responds quickly to a mineral deficiency, or to its correction, a relatively direct connection with the mineral factor is suggested but the mechanism of this effect remains obscure. It is not known whether there is an ion-specific structural influence on reaction systems, or whether rapidly occurring and disappearing metabolic stimulators or inhibitors have an intermediate effect. This is the point at which gas exchange analysis of deficiency symptoms must be extended by means of a refined chemical analysis; in this case, particular attention must be paid to possible changes within the energy-transferring systems. After insight has been gained into the connection between mineral nutrition and metabolism, it would then be important and especially rewarding to

compare the behavior of cells and their fractions on this new basis. Certain starts in this direction are already available for chloroplasts (11, 17, 56).

Finally, a critical reference must be made to the physiological behavior of cells in the course of the above-mentioned recovery experiments. It is easy to make the mistake of considering the recovery of metabolic functions from a mineral deficiency as a simple process of normalization. According to the hitherto available evidence, this is actually the case with the photosynthesis mechanism; in respiration, however, the situation is somewhat different, since the respiratory rate in some cases reaches at first markedly supernormal values in the course of recovery before settling down to a normal rate. This phenomenon is of special interest, although it complicates the study of the recovery process; therefore, it will be expressly mentioned again in the following sections.

Potassium.—The interference of potassium with the nitrogen metabolism of higher plants has been demonstrated again in the sunflower by Eaton (75). The hitherto available data on this question do not make it completely clear whether the relative enrichment of soluble organic (and inorganic) nitrogen fractions in relation to protein in potassium-deficient plants is to be considered as a clear-cut early symptom, ascribable to an inhibition of the protein synthesis, or whether it is due to a perhaps secondarily enhanced proteolysis. The dark green coloration at the onset of the deficiency, which was also noted by earlier observers, does not appear to be generally demonstrable by quantitative pigment analyses of deficient potato leaves [Bolle-Jones & Notton (39)]. This coloration argues against a primary disappearance of protein, since chlorophyll is in general, although not always, a good indicator for the protein level of the cell. There is a remarkable increase of the amide fraction in sunflowers, as well as an increase of ammonia salt, which latter is completely lacking in normal controls. It has been shown by Richards & Berner (234) that of the soluble organic nitrogen compounds in potassium-deficient plants, the more basic components are especially accumulated. These investigators made essential refinements in this observation with the help of paper chromatography. The accumulation of glutamine and asparagine in barley is especially noticeable. This is also true of putrescine, which is apparently especially characteristic of potassium deficiency and which has been found in unexpectedly large quantities [Richards & Coleman (235)]. Putrescine is apparently derived from arginine, which is also demonstrably present in large quantities. Arginine is particularly concentrated in flax, since there it appears not to be transformed into putrescine [Richards (232)]. On the other hand, decomposition in the case of red clover progresses, under the influence of abundantly present diaminooxidase, through putrescine to a not yet identified compound. Part of these results have been further confirmed by feeding experiments. According to these experiments, ornithine apparently cannot be considered as an intermediate product between arginine and putrescine. The increase in basic compounds should perhaps be considered as a regulation of the excess of anions which appears in potassium de-

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ficiency. These experiments indicate for the first time a specificity of potassium in regard to single steps of nitrogen metabolism; however, the degree of specificity requires further research. It may be parenthetically remarked that Alten & Orth (3) some time ago observed the inverse phenomenon in the potato, namely the raising of the arginine level after the addition of potassium, with a simultaneous tendency toward a lower level of the other organic nitrogen fractions.

In the carbohydrate metabolism of potassium-deficient cells there has been reported an increase of reducing sugar, together with at most a slight change in total carbohydrates [cf. Steinberg (264)]. In individual cases there are, however, exceptions and discrepancies; it is also not certain that an accumulation phase, as an early deficiency symptom, precedes the decrease of carbohydrates, as has been described anew in the case of the sunflower [Eaton (70)]. The situation here is complicated by the marked translocation of potassium, which is especially important in the case of potassium-deficient plants [Löhr (166)]. When an accumulation of carbohydrates occurs, it is probably in direct connection with an inhibition of protein synthesis. Reducing sugars can be increased by an enhancement of invertase activity, which has been demonstrated by Buzover (53) in potassium-deficient potato plants. Scheck (245) has found an increased activity (or quantity) of carbohydrases (β-amylase, invertase, β-fructosidase, and β-glucosidase) in several crop plants. This is also true of crude preparations of potassium-deficient cultivated yeast and moulds which, in addition, are higher active in catalase (128). The situation is complicated by the fact that the carbohydrate balance in cases of potassium deficiency is double-influenced by inhibition of photosynthesis and respiratory increase. This basic and evidently direct connection between potassium and carbohydrate metabolism can be demonstrated with special clarity on algae of the *Chlorella* type. Photosynthesis progresses here, in the case of increasing potassium deficiency, through all stages of inhibition, according to Pirson *et al.* (218); the direct effect of potassium manifests itself in short-term recovery experiments (cf. Table I) by a reversibility which has no relation to growth or chlorophyll formation. In cases of a more marked potassium deficiency there is no reversibility due to deeper disturbances. In a moderate degree of deficiency an addition of potassium brings about a rapid initial increase with a photosynthesis quotient ($PQ = O_2/CO_2$) of 1, followed by a slow recovery of the total metabolism with assimilation of the newly-won carbohydrates and nitrates ($PQ > 1$). A coordination of these gas exchange measurements with analytic data has not yet been made; hitherto only chlorophyll determinations are available. One metabolic symptom which is demonstrable in algae in various degrees of potassium deficiency is an unmistakable increase in respiration, which is also easily reversible before regeneration of growth. This characteristic of potassium deficiency has also been described in other species, such as *Hydrodictyon* (189) *Lemna* (217), and higher land plants (4, 106, 108, 229). In the last case, however, (4) a possible reversibility of potassium deficiency

was not examined. In heterotrophic organisms where the counteractive effect of photosynthesis is lacking, the high respiration rate in cases of potassium deficiency results in a worsening of the metabolic balance [Rippel *et al.* (236)]. In *Chlorella* the oxidative assimilation of glucose is, under conditions of darkness, reversibly inhibited by potassium deficiency [Daniel (63)]. In the large cells of the green alga *Hydrodictyon*, which Neeb (189) introduced as an experimental object in this field in order to show relations between cellular morphology and physiological deficiency symptoms, potassium deficiency brings about an impoverishment of cellular substances as a result of the inhibition of photosynthesis and an increase in respiration, in contrast to phosphorus and nitrogen deficiencies. In this case the remnants of the degenerated chloroplasts remain rich in chlorophyll. In nitrogen-deficient cells, the plastids are reduced in size, and are accompanied by a chlorotic bleaching. In weak light, potassium deficiency manifests itself in the case of *Ankistrodesmus* (218) in a shifting of the compensatory light intensity toward the higher values. Here the carbon balance can be turned in a short time from the negative to the positive side through the addition of potassium. This recalls the old claim that potassium fertilization is especially efficacious in weak light [cf. (241)]. On the other hand, Schwabe (250) has demonstrated an increased sensitivity to strong light in sporophytes of bracken, the biochemical behavior of which, in case of potassium deficiency, is similar to that of the higher land plants which were earlier examined for the same characteristics by Gregory and his school. The effective range of light intensity is evidently considerably narrowed by potassium deficiency.

That the carbohydrate balance in potassium deficiency is also early and specifically disturbed in cereals can be indirectly supported by data from Nicholas (195), who says that the minimum amount of potassium which will produce an optimal grain yield is higher than the amount of potassium at which visible deficiency symptoms appear. Potassium deficiency also seems to be relatively easily reversible in higher plants, at least at the beginning. O'Rourke & Marshall (204) have recently stated that the inhibition of growth in young tung trees can be rapidly corrected in the case of potassium deficiency, but more slowly in the case of magnesium deficiency.

The characteristic increase of respiration in potassium-deficient cells may represent mainly an "idling" effect comparable to the supernormal respiration which is observable during the uncoupling of respiration from energy transfer by 2,4-dinitrophenol [cf. Simon (255)], or in the "climacteric rise" of ripening fruits [Pearson & Robertson (207, 208); Millerd, Bonner & Biale (181)]. A more precise examination is necessary to determine the role of potassium *in vivo* in the ATP/ADP system. Lardy (153) has compiled enzymological data which indicate that potassium is involved [cf. (42)]. Inhibition of CO_2 consumption (with simultaneous enhancement of phosphorylation), as well as increases through the addition of potassium, are known to take place in muscle preparations (43) and mitochondria (223). This shows how difficult it is at present to reconcile evidence on an enzy-

mological or mitochondrial basis with the behavior of intact cells. It has been shown by observation of the particularly sensitive system of bacterial luminescence that potassium effects *in vivo* can also manifest themselves quite variously in the same object, depending on external conditions. According to Schneyer (247) this system is protected by potassium from irreversible inactivation about 30°C.; it is reversibly hindered within the range of optimal luminescence (22°C.), but is favored by an addition of potassium at lower temperatures. These differences are attributed to slight changes of structure in the native proteins which control the luminescence.

While on the one hand the potassium level of the cell controls the processes of photosynthesis and respiration to a high degree, on the other hand the extent of these metabolic processes is important for the uptake of potassium. Rathje (226) has found that in respiring and fermenting yeast suspensions the uptake of potassium parallels the CO_2-production and hydrogen ion concentration; thus, he assumes that K^+ ions are exchanged for H^+ ions. It is remarkable that photosynthesis in *Chlorella* and *Lemna* also enhances the uptake of potassium, according to the degree of intensity; here a tendency toward acidification, also during photosynthesis, is assumed to be the causal factor.

An inhibitory effect of potassium deficiency upon the formation of cell membrane, which, from a practical point of view, has often been discussed as the cause of insufficient standing ability in deficient cereals, has been established by the comprehensive examinations of Mulder (185) on the influence of mineral factors on "lodging." The specificity of such a potassium effect is, however, uncertain; in particular, excessive doses of potassium have proven to be without effect upon firmness. Potassium deficiency reduces the microscopically observed thickness of the walls of sclerenchyma cells, but the lignin content of the potassium-deficient tissue tends to be raised. Thus, we see that this long-standing problem cannot, from a physiological point of view, be considered solved.

Sodium, Rubidium, Cesium.—The problems of substitution and replacement are of special interest in analyzing the function of potassium. A partial substitution by sodium, which, heretofore, has been mainly considered from an agricultural point of view, was discussed in a symposium, the contributions to which were published in a special issue of "Soil Science" (28). Harmer *et al.* (112) characterize the reactions of numerous culture plants to sodium according to the following scheme: (*a*) slight or no reaction, also in the case of insufficient addition of potassium, e.g., in corn, lettuce, onions, potatoes, soya, and spinach; (*b*) weak to moderate reactions (partial substitution) at potassium deficiency, e.g., in barley, oats, mustard, and tomatoes; (*c*) weak to moderate reactions at sufficient potassium supply, e.g., in cabbage, peas, oats (156, 297), cotton (58, 172), flax (154), potatoes (156), radishes, and wheat; (*d*) strong reaction (i.e., marked stimulation of growth) with sufficient potassium supply, e.g., various strains of beet, and celery. This list [cf. (277)] shows that the claims of the various authors do not com-

pletely coincide. Although the effect of sodium is markedly susceptible to external influences, the most favorable effect seems to occur generally in those cases in which the maximum amount of sodium is absorbed with a minimum depression of the potassium content (154). The effect of sodium is supposed to be at least partially due to an intracellular synergism of potassium and sodium, and furthermore, in the last-named groups, to a specific enhancement by sodium. Some authors speak here of a nutritional effect without being able to make any definite statements about its nature. Naturally, there can be no certainty of the real necessity of sodium on the basis of the available data. In cases where sodium enhances the uptake of potassium and reduces that of calcium (141, 154), it may be assumed that the ion balance is only indirectly influenced. In single cases, two-thirds of the normal potassium requirement can be replaced with sodium without causing growth disturbances [Gammon (95)]. From the agricultural point of view, a favorable effect of sodium on the development of the root system of alfalfa [Cope *et al.* (59)] and beets [Scharrer *et al.* (244)] has been pointed out. The whole problem is ripe for analysis by the more subtle methods of plant physiology. In this regard it is significant that there is also an evident intercellular partial substitution of sodium for potassium in *Aspergillus* [Steinberg (263); Culot (62)]. On the other hand, in unicellular algae potassium does not share its marked effect upon growth and metabolism with sodium (209, 218).

A partial substitution of potassium by rubidium in barley has been observed and thoroughly examined by Richards (230, 231) in the range of low potassium supply. A specific increase in growth by rubidium in the presence of potassium, for which certain evidence is available in green algae (210), is lacking in barley. The substitution is demonstrable only in the case of limited potassium supply; here, it is, however, quite clear. This substitution applies to the partial function of potassium in the regulation of the ion balance, and especially to the avoidance of an excessive uptake of phosphate and NH_4^+ ions. In addition, there is a replacement effect within cell metabolism which prevents the accumulation of amino acids and amides (e.g., putrescine) in potassium deficiency (232). The same result can be obtained with sodium and even with lithium. Potassium-deficient plants are able to assimilate putrescine when it is supplied to them together with rubidium or sodium; however, potassium itself is more effective. Higher dosages, in the concentration which is customary for potassium, produce a toxic effect of rubidium in higher plants; its symptoms are different from those of potassium deficiency. Partial substitution is therefore only possible within a limited range of concentration, which varies from object to object according to the degree of toxicity. In suitable dosages partial substitution has also been demonstrated in *Lemna* cultures [Pirson & Kellner (215)]. Injection experiments by Kassel & Kopac (140) on amoebae, undertaken to determine fractionation media with optimal ion balance, showed no intraplasmatic disturbances so long as rubidium in sufficiently low concentrations was added in place of potassium.

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The effect of alkali cations in enzyme activation *in vitro*, which has been examined recently for oxidative phosphorylation in rat muscle preparations and for the respiratory increase in rat liver mitochondria (223), appears to be expressible by the series $K = Rb \geq Cs > Na \geq Li$. Similar relationships may be assumed to hold true for metabolic reaction steps *in vivo*. Lardy (153) reported on this question, and has stressed the primary participation of phosphorylations in such cases. Lwoff & Ionesco (169) have found that the relationship of increase of oxidative decarboxylation of malate and pyruvate in bacteria is $K = Rb > Cs$. The short-term recovery effects of photosynthesis and respiration in *Chlorella* also follow this series (210). In the growth of these algae the series diminishes to $K > Rb$; in any case, however, thick cell suspensions can be obtained with rubidium if potassium is excluded as far as known purification methods permit. The possibility that, also in this case, rubidium causes only a partial substitution, in addition to a basic effect of potassium, has been tested by a series of tests at various levels of concentration (142, 210, 215). A possibly required minimal dose of potassium would, in any case, have to be so slight that the potassium could be regarded here only as an extreme micronutrient. At present there is no evidence for such a completely specific trace effect of potassium. The green alga *Ankistrodesmus*, which is closely related to *Chlorella*, does not have an innate ability to exchange potassium for rubidium, but can acquire it through mutative adaptation [Pirson & Kellner (215)]. The mutation apparently does not apply to replaceability in itself, but rather to resistance to the toxic effect of rubidium, which is the continuously dominating factor in the rubidium effect on higher plants and also on algae cells with a more complicated structure (*Hydrodictyon*). The algae cells which have become adapted to rubidium are also more resistant to the toxic effects of cesium; resistance to rubidium and cesium may be considered as a manifestation of the same physiological factor in the cell, in contrast, e.g., to the resistance against copper, which can be separately acquired by mutation (215). Cesium, however, can in no way replace potassium in growth. Rb-adapted cells have a higher respiratory rate and thus a less favorable carbon balance in a Rb medium, but not in the normal nutrient solution. Perhaps this is the main reason for the permanent retardation of growth of these cells in Rb-medium compared with normally cultivated control cells (142).

In addition to, or as a result of, its toxic effect, rubidium also exerts morphogenetic influences. In nonadapted *Ankistrodesmus* cultures there appear, in a rubidium medium, multi-nuclear giant cells with an "anarchic" growth. In this case, cell division stops, whereas mitosis and production of plasmatic substance continue. The giant cells may quickly split up by subsequent division after being transferred to a normal medium. Cells which once have become adapted to rubidium show very few or no morphological abnormalities. Richards (233) has observed evidently specific morphological symptoms of rubidium treatment in higher plants. As a result of local stimulation of cell division, the petals of flax develop characteristic keel-like protuberances over the midrib. The formation of this structure which is limited

to certain flax types and the genetic basis of its reaction pattern are still being examined.

The toxic effects which alkali chlorides in one-salt solutions exert on the growth of seedling roots, increase in the series $Na < K < Rb < Cs < Li$ (160); in some cases sodium is more toxic than potassium, Effects of this type and their diminution through the application of various combinations of mono- and bivalent cations are, from the standpoint of functional analysis, no more indicative at present than they have been in the past, since there is a concatenation of too many factors. Libbert (160) has attempted to separate them and judge their respective importance.

Calcium (*Strontium, Barium*).—The classical ion antagonism between potassium and calcium is doubtless a phenomenon of basic physiological significance, and therefore has been frequently investigated [cf. (160)]. But this has tended to distract attention from the specific effects of these ions. A careful examination of the latter, however, should throw new light particularly on the part played by calcium. Certain indications are already given by the wide variations in quantitative calcium requirement in micro-organisms and higher plants. Unicellular green algae require calcium in minimal quantities (203, 261, 288); the requirements of blue algae are not yet clear (98, 99). Under certain nutritional conditions calcium is apparently not necessary in bacteria (105), but this is a subject which requires further examination [cf. (68)]. Green algae with large or sharply differentiated cells (189) have, like the mycelium-forming fungi in general, higher requirements, which seem to approach partially the requirements of higher plants. It may be assumed that, in marked cases of cell elongation, calcium takes over an additional and specific function. Burström (51, 52) has actually shown that a functional tripartite division of the calcium requirement in wheat roots has to be taken into consideration. A minimal amount (10^{-6} *M* in an external medium) is necessary for cell division; the requirement necessary for cell elongation lies about ten times higher, and still higher dosages are useful or even necessary for the detoxification of H^+ ions. It may be remarked that this antagonistic effect of Ca^{++} and H^+ ions in algae has already been observed at the level of micronutrient concentration (209, 261). Burström has concluded from extensive examinations of the specific effect of calcium on root growth, a symptom which has long been known, that calcium represents a kind of antiauxin, and as such enhances the cell elongation in roots. While, then, auxin is supposed to provide for the loosening of the cell wall texture, calcium does not affect the plasticity of the cell wall, but rather interferes in the production of elasticity at the later phase of intussusception. Calcium also aids in enhancing the nitrate uptake in roots. Since a lower nitrogen level favors cellular elongation [cf. Bosemark (41); Pirson & Göllner (213)], an indirect growth enhancement by calcium deficiency via nitrogen metabolism is possible. However, the direct, i.e., the opposite effect of calcium deficiency appears to predominate.

It is well-known fact, recently confirmed (224, 238), that aging cells

and tissues show an increase in the Ca/K quotient. According to the conventional view, this shift of ion balance results in a lessening of intraplasmatic hydration. A disappearance of "lyophilic colloids" is supposed to be connected with the aging process [Zhukov (310)]. This shift in ion balance is naturally a result, rather than a cause, of aging; the cell-physiological and protoplasmatic behavior of aging root cells of *Lemna*, which has been examined (217) differs in several respects from the behavior of younger cells with beginning potassium deficiency or calcium excess.

Antagonistic effects of K^+ and Ca^{++} ions have been examined by Kachmar & Boyer (137) on an enzymological level. The enzyme for transphosphorylation from phospho-enol-pyruvic acid to ADP is activated by Mg^{++} and K^+ ions (or NH_4^+ and Rb^+ ions), but strongly inhibited by Ca^{++} ions. This inhibition, in turn, can be partially counteracted by high dosages of K^+ ions, but even then there remains a specific Ca^{++} inhibition. It is not yet understood whether in other or all cases of K^+-activated enzyme reactions Ca^{++} interferes in a similar way. Enzyme activation by Ca^{++}, as has been described by Gorini (104) in proteinases, may be an indirect effect, in that Ca^{++} removes such inhibitory factors as surplus anions. A striking aid of Ca^{++} in the mechanism of active K^+-uptake has been shown by Overstreet *et al.* (205) in barley roots.

As far as the effect of calcium lies in a pure interfacial reaction, it might be elucidated by model experiments of the type which Netter (193, 194) has recently performed.

A complete substitution of strontium for calcium is, according to Walker (288), possible for the extremely slight calcium requirements of *Chlorella*. The same holds true for the aquatic phycomycete *Allomyces* [Ingraham & Emerson (134)]. In such a complete substitution, which represents the maximum known replaceability in growth experiments, perhaps only a very simple, surface-bound effect of Ca^{++} (or Sr^{++}) is involved rather than a deeper interference with the intra-plasmatic regulation of the metabolic processes. A partial replaceability Sr/Ca in the removal of proteinase inhibition in bacteria has been observed by Gorini & Audrain (105). Basu (24) reports even a substitution effect of barium in fungi (*Chaetomium*). Such experiments are naturally affected by the low solubility of the sulfates.

Magnesium.—It is well known that in cases of magnesium deficiency disturbances appear which are independent of decreasing chlorophyll content; they may precede the latter in the early stages of deficiency. So far it has been very difficult to resolve these symptoms into single factors. Magnesium is the most effective activator within a vast complex of enzyme reactions [cf. (173)]; also, it undoubtedly functions in a complicated manner as an activator in the cell. Therefore, it would be difficult to isolate the single effects of magnesium by physiological experiments. It must be kept in mind that magnesium can also develop inhibitory effects, due to its special tendency to complex formation with active sulfhydryl groups. Ramakrishnan & Martin (225) have found, for example, that even the citrate-forming "con-

densing enzyme" from *Aspergillus niger* is inhibited by Mg^{++}. *In vitro* manganese seems to be more active than magnesium in regard to the enzymes of the citric acid cycle (173). Najjar (188) has described activation reactions in which calcium is superior to magnesium.

Some time ago Fleischer (88) concluded that magnesium plays a special part in photosynthesis from the fact that photosynthesis in magnesium-deficient *Chlorella* is more strongly inhibited than is chlorophyll production. Later Kennedy (143) attempted, through experiments with flashing light, to localize this disturbance in the "Blackman reaction." This application of the concept of Blackman factors to the conditions of mineral deficiency appears not to be quite successful, since here also disturbances must be taken into consideration which relate to the substantial basis or structure of the photosynthetic apparatus without interfering primarily with the chemical mechanism of the latter. It has actually been demonstrated that the inhibition of photosynthesis in *Ankistrodesmus* through magnesium deficiency is a secondary effect, since it cannot be rapidly corrected, i.e., it continues for some time even after magnesium has been added (Pirson *et al.*, cf. Table I). A slight resumption of photosynthesis after addition of magnesium was demonstrable in recovery experiments only in a few cases with very slight magnesium deficiency. Such a lasting disturbance, even in cases of a quantitatively slight inhibition of photosynthesis, may be attributed to an interference in protein synthesis. In the earlier deficiency phases before the sudden onset of extreme chlorosis there may, however, also occur an irreversible structural change. Or there may be a blockage of reaction loci, in which magnesium normally functions as an activator, by the formation of stable and inactive complexes; newly-added magnesium, as weaker complex-former is no longer able to eliminate those complexes. Respiration in deficient cells is slightly and also irreversibly increased.

Finkle & Appleman (87) have directed attention to the fact that magnesium deficiency interrupts the division in *Chlorella* cells without immediately bringing about a general inhibition of synthesis. As a result the cells grow to abnormal sizes. A similar situation is true for *Ankistrodesmus* (218). However, it must be mentioned that a separation of cell division and cell growth also occurs in other deficiencies, e.g., in *Ankistrodesmus* with potassium deficiency or strong phosphorus deficiency. According to unpublished findings of the present writer, the behavior of various algae strains is not uniform in this regard, so that generalizations on this subject must be guarded against. Finkle & Appleman (86) have also traced the activity of catalase in magnesium deficiency in its relation to chlorophyll formation. Within a narrowly limited deficiency range it can be shown that cessation of chlorophyll formation leads to an increase of catalase activity. A common metal-free porphyrin precursor of catalase and chlorophyll is evidently, in cases of a low magnesium level, shifted over entirely to a hemin route. A slight quantity of magnesium appears also, however, to be necessary for catalase formation. This behavior in algae is almost exactly the same as that of barley seedling

leaves grown under magnesium deficiency [Appleman (5)]. Here may also be mentioned the findings of Webb on *Clostridium welchii* [cf. the synopsis in (296)], which, like other gram-positive rods, shows an inhibition of cell division and a resulting thread formation in cases of magnesium deficiency. This effect is apparently attributable to an inhibition of protein synthesis, without any simultaneous disturbance in the DNS level (nuclear system). Gram-negative bacteria do not react so sensitively to magnesium deficiency.

Attempts to replace magnesium with beryllium in deficiency cultures and in metabolism experiments have hitherto been unsuccessful (218). A partially reversible inhibition of photosynthesis does not appear until the concentration of $BeSO_4$ reaches 10^{-2} *M*, whereas in enzyme experiments (alkali phosphatases) much lower concentrations bring about inactivation (279). In this connection it is interesting to note that Steinberg (263) has demonstrated in *Aspergillus* a partial substitution of beryllium for magnesium in the range of slight magnesium doses. Mitosis in animal cells is inhibited by concentrations of beryllium from $2{,}5 \cdot 10^{-3}$ to 10^{-6} *M* (55).

For higher plants there is but little recent information on the physiological effect of magnesium. Magnesium deficiency is relatively difficult to correct (204); in celery the chlorosis symptoms do not disappear until eleven weeks after foliar application (220). Sensitivity to magnesium deficiency varies greatly from plant to plant and is conditioned by a single gene. Hewitt (118) has shown that an acetone-soluble nonchlorophyll magnesium fraction in beet leaves [corresponding to a similar ether-soluble magnesium fraction which has been described already by Smith (258)] is accumulated by chlorosis-producing excess doses of Mn^{++} and Zn^{++}. Apparently, this fraction, which is considered to be a precursor of chlorophyll, is prevented from further assimilation. The situation appears to be more complicated in cases of other excess chloroses. Since the problems of salt uptake will not be further treated here, short mention may be made of the close positive correlation between the uptake of magnesium and that of phosphate, which results from foliar analysis of apple trees after various manuring measures [Mulder (184)].

Nitrogen.—Since the classical work of Warburg & Negelein in 1920 (292) the question of the intermediate products of nitrate reduction in green algae has not been raised again. Whereas, Warburg considered nitrite to be a pathological side-product appearing only in anaerobiosis, Kessler (144, 145) has shown that under conditions of darkness an experimental separation of reduction can be interrupted at the nitrite stage by a moderate lowering of pH within the physiological range, and that nitrite actually appears in large quantities. The C_H tolerance of nitrite reduction differs according to the species or strain within the individual green algae (146). This is demonstrated by a continuous series beginning with forms which produce nitrite at pH 7 and leading up to those which even under pH 3 still completely reduce nitrate, but which will, in anaerobiosis, accumulate nitrite as a genuine intermediate product. Nitrite accumulation within a favorable pH range,

as is also the case with addition of nitrite, leads to a marked increase in respiration; this should not be considered as "idle" respiration, but rather as representing a regulatory effect, since with its help, and under suitable experimental conditions, nitrite is reduced. Kessler (147) has further found that 2,4-dinitrophenol, which separates the ATP-controlled, energy-consuming reactions from dissimilation processes, also leads to accumulation of nitrite. Thus, nitrite reduction appears to be connected with ATP, whereas the formation of nitrite from nitrate, as a process with lower energy requirement, shows this connection, if at all, only to a very slight extent. In light the equilibrium between nitrite formation and nitrite reduction is maintained under the same conditions. Therefore, it appears possible, although not yet proved in these experiments, that the photosynthetic apparatus interferes directly in the process of nitrate reduction.

Further experimental support has recently been offered for this widely-discussed assumption. Van Niel, Allen & Wright (201) have found in *Chlorella* under strong light a considerably larger evolution of O_2 with constant CO_2 uptake in the presence of nitrate than in its absence; they therefore assume a direct nitrate reduction in the photosynthetic mechanism. It is striking that the photosynthetic apparatus, which is maximally burdened by CO_2 assimilation, could still facilitate nitrate reduction without bringing about a competition between CO_2 and nitrate. In weak light the photosynthetic CO_2 uptake is lessened during nitrate reduction [cf. Cramer & Myers (61)]. This may be the result of competition between CO_2 and nitrate for the photosynthetic reducer, or it may be that a nitrate reduction, at least not directly dependent upon light, supplies extra carbonic acid which counteracts the unchanged CO_2 consumption in photosynthesis. Nitrogen-deficient cells with inhibited photosynthesis react in strong light to an addition of nitrate with a rapid decrease of apparent CO_2 uptake and a slightly raised production of O_2 [Pirson & Wilhelmi (219), Wilhelmi (302)]. After darkening, nitrate reduction here can proceed to the same extent as before. The mechanism of nitrate reduction in darkness is therefore not automatically stopped. It may be indirectly augmented by light under certain conditions (e.g., by the increased quantity of reducing photosynthetic products). Davis (66) found in the absence of CO_2 a considerable nitrate reduction in light only when he gave glucose and phosphate to *Chlorella*. This can be most easily explained by a nitrate reduction under direct help of glucose or one of its decomposition products as a hydrogen donor. Nitrate reduction in green algae is thus still a complicated problem requiring in many respects further elucidation. Syrett (272) recently threw some light on this situation. Evans & Nason (83) succeeded in effecting a nitrate reduction to nitrite with illuminated chloroplast fragments, when TPN and nitrate reductase from soy bean leaves were present. This, however, does not clearly prove an equivalent light reaction to be true in the living green cell. Mendel & Visser (177) have shown most clearly on tomato leaves that a specifically light-dependent reduction of nitrate must also be reckoned with in higher

plants; the nitrate reduction in tomato leaves, as measured by the incorporation of nitrate tracers (N^{15}), is in light, in contrast to that in darkness, independent of carbohydrate content and insensitive to iodine acetate. Yoshimura (309), on the other hand, has found that in Lemnaceae the ammonium uptake is accelerated by light, but that nitrate reduction is not; an indirect light effect via an increase of O_2 pressure in the tissue, is supposed to favor ammonium assimilation, but hinder nitrate reduction.

It may be concluded from many experiments on autotrophically-fed green algae that nitrate and ammonium salts can serve as practically equivalent nitrogen sources [cf. most recently, (218)]. Even when the carbon supply does not inhibit growth (heterotrophic cultures), no difference is noticeable so long as acidification does not appear as a disturbing factor in the ammonium cultures (32). If nitrate and ammonium ions are simultaneously added, the latter are taken up preferentially, as has recently been confirmed by Schuler, Diller & Kersten (249) on *Chlorella* through the use of N^{15} tracers as a nitrogen source. However, nitrogen-deficient cells show the gas exchange characteristics of nitrate reduction in manometric experiments in light when ammonium nitrate is added [Wilhelmi (302)]. The biochemical equivalence of ammonium and nitrate nutrition cannot be easily established even if the visual appearance of higher and lower plants would seem to indicate it; the plants used for comparison can still show certain differences within the range of physiological tolerance. Kalinkewitch (138) claims that nitrate-fed plants tend to accumulate organic acids, while ammonium-fed plants tend to form reduced products.

The most important result in the field of nitrate reduction from a biochemical point of view, namely the isolation of nitrate reductase [cf. (173)], has been put on a broader basis by Evans & Nason (83) and Nason *et al.* (197, 198). Yet, it is still not known which metal activates the nitrate reductase of green plants, which has been isolated as flavine-adenine-dinucleotide from soy beans and other higher plants (83), and which differs in certain respects from the molybdenum-dependent *Neurospora* enzyme. As a pure preparation the enzyme reduces nitrate quantitatively only down to the nitrite step. TPNH, like DPNH, is perhaps also engaged in nitrate reduction.

The stages of nitrite reduction, which is energetically the more difficult part of the total process, are still not clear. Steinberg (265, 266) has found in orientation experiments that tobacco seeds (like one *Aspergillus*), with careful feeding can assimilate in addition to nitrate and nitrite, also nitrohydroxylaminate, but not hydroxylamine or hyponitrite, as an exclusive nitrogen source; thus, nitrohydroxylaminate may be considered as an intermediate product. Kluyver & Verhoeven (148a) have suggested a reaction scheme which links the normal assimilatory path of nitrate reduction with the dissimilatory one (dentrification). Both ways branch from a hypothetical radical-intermediate $=NOH$; NH_2OH is placed an an immediate precursor of ammonia. Kumada (149) has found that the ability to assimilate nitrates varies in the single parts of Leguminosae seedlings, and also changes with

age; cotyledons, for example, reduce only to nitrite. This shows anew the physiological complications which are produced by the morphological differentiation of the cormophyte.

In passing over the further problems posed by the metabolism of organic nitrogen compounds, there should still be noted certain recent views which have been provided by the examination of metabolism in nitrogen-deficient plants in the question of the function of inorganic nitrogen sources. Of general importance is the remark of Virtanen (284) that in nitrogen deficiency at first the ability to form adaptive enzymes is reduced, whereas the fundamental complement of vital enzymes is maintained; the synthesis of the latter has an absolute priority. Even when inactivation occurs, the ability to reactivate promptly the enzymatic processes is maintained as a striking characteristic of nitrogen deficiency—a point of great ecological importance.

If we turn back to green algae, we find that at first their respiration is either increased or normal, according to the species or strain used, but in cases of marked nitrogen deficiency it is always low (209, 218). Photosynthesis decreases sharply, from a critical nitrogen level on, at approximately the same rate as chlorophyll; in strong light chlorophyll is subjected to a decomposition which far outweighs the disappearance of protein [Aach (1)]. Subsequently, fat appears, often in astonishing quantities, as an assimilate which, in turn, is confined to the strain; sometimes it appears together with secondary carotinoids. An addition of nitrate produces, in cases of slight nitrogen deficiency, only a slight increase in photosynthesis, which is already strongly inhibited (49, 218); this is directly followed by a resumption of nitrate reduction and assimilation (cf. Table I). This latter process controls the behavior in recovery from more advanced nitrogen deficiency. Gas exchange in light is much complicated by the marked response of respiration during the process of recovery; this has been separately examined in two laboratories. In extremely fatty, orange-red *Ankistrodesmus* cells all usable nitrogen sources function in this way, but with differences of rate and quantity. If an equal amount of nitrogen is added, stimulation occurs in the first hours of recovery according to the following scheme: ammonium > urea > nitrate > amino acids and amides (49). Therefore, a general principle for all nitrogen sources must be developed on a graduated scale to deal with the activation of respiration. Syrett (270, 271), who has particularly traced the effect of ammonium and has carried out parallel analyses of nitrogen and phosphorus fractions, assumes as a working hypothesis, with reference to the literature, that the active synthesis of amino acids and amides stimulates respiration by making abundantly available phosphate acceptors (ADP), which, earlier, had a limiting influence (272). Any discussion here must take into account the fact that respiration, during recovery, reaches, during the initial stage, a rate several times its normal value but decreases again after a while, approaching the respiratory rate of normal cells long before the reserves are depleted, in darkness as well as in light (49). The maxima of respiration and bulk of new cellular syntheses evidently do not occur together. Here, there

may be at first a considerable "idling" of the respiration, until the mass of synthetic reactions is coupled with the respiration, with a deceleration of the latter. Actually, the respiration of nitrogen-deficient cells is stimulated by ammonium salt in a similar manner as by the uncoupling agent dinitrophenol (272). Warburg & Negelein (292) remarked in 1920 that an examination of metabolic reactions in nitrogen-deficient algae would be an especially rewarding task; today this remark is just beginning to be confirmed.

The increase of respiration in nitrogen-deficent cells, at times to supernormal values, after addition of ammonium and other nitrogen sources is not confined to algae, but seems to be rather a general phenomenon which has been demonstrated on heterotrophic micro-organisms as well as on the green and colorless parts in higher plants, [cf. (111, 213, 272)]. Extensive examinations have been made on young barley plants by Folkes, Willis & Yemm (91), who have also tried to explain the effect of ammonium as a mutual adjustment of synthetic activity and carbohydrate catabolism, in the same way that Syrett (272) has done for green algae.

Phosphorus.—In a series of studies Eaton (71, 72, 74) has undertaken an analysis of metabolism in three different phosphorus-deficient plants (sunflower, soy bean, black mustard); he did not achieve uniform results. Soy bean show a more marked disturbance of nitrogen metabolism as manifested in an accumulation of total soluble nitrogen and of amides. Inhibition of synthesis takes place at the amide stage, but does not at the stage of nitrate reduction. Soy bean leaves become definitely chlorotic after passing through early chlorophyll accumulation which, as a general symptom phosphorus deficiency, is well known but not yet understood, and has even been detected in unicellular green algae (218); chlorosis is absent in sunflowers and black mustard, and in the same way these latter show no marked influencing of their nitrogen metabolism in the range of more pronounced phosphorus deficiency. Therefore, the accumulation of carbohydrates (partially in the form of starches) which is generally observable in the middle phase of phosphorus deficiency, especially in stalks, cannot be traced back exclusively to a cessation of protein synthesis; it is at least partially the result of shoot stunting or of a relatively high leaf/stem ratio (fresh weight), i.e., of the ratio of assimilate-suppliers to assimilate-storers. Photosynthesis appears not to sink to low values until comparatively late. Lapshina (152) reports that phosphorus deficiency in oats reduces total sugars and protein; the nonprotein nitrogen in the roots assumes abnormally high values. Phosphate applied by spraying soon reinitiates nitrogen uptake, protein synthesis, and carbohydrate formation. Thus, the picture of the biochemical situation in phosphorus-deficient plants is not yet completely clear.

A lowering of the shoot/root ratio (71) may be considered as a general characteristic common to both phosphorus and nitrogen deficiencies. This phenomenon may even be observed in *Lemna*, which is morphologically quite different (213). Apparently this phenomenon is a result of the sinking of the auxin level in the whole plant. Pirson & Göllner (213) have compared the

symptoms of phosphorus and nitrogen deficiencies in *Lemna* roots and have ascertained that the two conditions are to a great extent identical: initial increase in root growth, larger cells, higher plasma viscosity (as measured by the so-called "time of plasmolysis"), higher osmotic value, lowered CO_2 consumption. The same characteristics are altered in opposite direction in cases of potassium and calcium deficiencies. There are also some common features in the behavior of micro-organisms with nitrogen and phosphorus deficiencies [Neeb (189)]; therefore it is easy to attribute a part of phosphorus-deficiency symptoms to disturbances in nitrogen metabolism. A problem, the biochemical causes of which have not yet been clarified, is the strong accumulation of fats in phosphorus-deficient algae (and fungi), and the enhancement of sugar conversion, which appears in *Chlorella* at a low phosphate level in the course of oxidative assimilation [Daniel (63)]. Photosynthesis in *Ankistrodesmus* is inhibited in strong light at the beginning of phosphorus deficiency and can be quickly reactivated in recovery experiments (cf. Table I). The behavior here resembles that in potassium deficiency, except that, in contrast to the latter, part of photosynthesis can be reactivated even in cases of extreme deficiency. In weak light there is apparently no primary inhibition; according to Lindeman (162), photosynthesis in *Lemna* in cases of phosphorus deficiency is also inhibited and reactivated only in strong light. It has not yet been determined whether the reversible chlorophyll-independent inhibition of photosynthesis is due to a structural or to a chemical disturbance, although the latter assumption is more likely in view of the numerous indications of the participation of phosphorylations in the photosynthetic process.

The role of phosphates in energy transfer, especially in the photosynthetic process, is presently the object of extensive experimental and theoretical studies. In this article it is impossible to go into the biochemical aspects of this problem, since this would involve an extensive treatment of special questions of photosynthesis, and of the methodical difficulties in analyzing phosphates and in judging the single fractions. Thus, mention may only be made of several synoptic reports (94, 269) and original works on special aspects of the problem (109, 256, 295, 305). Arnon (10) has given a general survey of the role of phosphorus in plant physiology and biochemistry. The most important point of reference for the part played by phosphate in photosynthesis is furnished by the determination of a light-dependent, short-term change in the level of inorganic phosphate (139) or, vice versa, of soluble organic phosphates; this determination was also made by use of phosphorus tracers (109). Direct biochemical observation of a specific light-dependent phosphorylization $ADP+PO_4=ATP$ is not yet possible in living green cells; however, Strehler (269) has produced a biological demonstration of this with his elegant firefly test. Energy-rich phosphates have been produced in light with the help of isolated chloroplasts [Arnon *et al.* (13)]. Simonis & Schwink (257) have added a paper chromatographical proof of a compound resembling ATP in *Helodea* and *Ankistrodesmus* to the already available data on the occurrence of adenosine phosphate in green cells.

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There is a growing interest in metaphosphates and polyphosphates (133, 246), with which cells are possibly able to store up an energy reservoir with the help of not directly used energy-rich primary phosphate compounds (ATP) [Hoffmann-Ostenhof & Weigert (127)]. The determination (isolation) of this phosphate fraction is methodically difficult; the best possibility here is a combination of preparative and chromatographical manipulations (304). In plants, metaphospates have so far been demonstrated only in microorganisms (133), i.e., in heterotrophic ones (moulds, yeasts) and recently also in autotrophic algae such as *Chlorella* (129), *Acetabularia* (267), and *Hydrodictyon* (unpublished observations in the laboratory of the present writer). There are clear indications that in light the metaphosphate and polyphosphate level in algae rises, and in darkness decreases in favor of orthophosphate [Stich (267); Wintermans (304)]. Stich has attempted to coordinate these changes in *Acetabularia* with the growth rate; Hämmerling & Stich (110) have shown on the same much-tried object, with phosphate tracers, that nuclear cells, as well as still-living anuclear cell fragments, irreversibly bind phosphorus, especially in light, and employ it in their energy economy. In any case, in the process of growth they apply the phosphate in the long run quite economically. A further biochemical study of this general biological principle would be of great interest.

As yet nothing is known about the finer changes which appear in the various phosphorus fractions after the withdrawal of phosphorus. For soy beans there are data indicating a relative increase of the inorganic fraction in comparison with the organic [Bagaev (20]. With normal nutrition, organic phosphorus is predominant in roots, mineral phosphorus in leaves. Eaton (71, 72, 74) has found in his phosphorus-deficient plants a relative increase of 70 per cent alcohol-soluble phosphate in comparison with insoluble phosphate, while there is naturally a marked sinking of the total phosphorus level.

Sulfur.—Since Gilbert's detailed review on sulfur nutrition (102) there have appeared only a few publications dealing directly with sulfur function and the points at which it influences metabolism in plants. As in so many cases, the results of visual observation and of the analysis of the main constituents and their standard fractions are not very encouraging from the physiological point of view Eaton (69 to 75) has studied the same plants for sulfur and phosphorus deficiency. Many characteristics of the deficient plants indicate a disturbance in the nitrogen supply (marked chlorosis, tendency to an increase of soluble nitrogen, of starch and of sucrose, tendency to a decrease of proteins and reducing sugars). Similar experiments however, on cotton by Ergle & Eaton (80), as well as by Ergle (78, 79) have brought to light characteristic differences in the behavior of sulfur-deficient and nitrogen-deficient plants. It is particularly remarkable that the protein sulfur of chloroplasts is, in comparison with other sulfur fractions, less affected by sulfur deficiency. The chloroplasts have evidently an advantageous position in the competition for the remaining sulfur. However, one would perhaps go too far in linking this observation directly with a role attributed to sulfur as a constituent of thioctic acid or similar cyclic S-S compounds in photo-

synthesis [cf. Calvin (54)]. In nitrogen-deficient plants nitrogen is transferred from older leaves to the younger parts by proteolysis, whereas in sulfur-deficient plants such a reassimilation of protein sulfur does not appear to take place (79). This difference results in a different chlorosis gradient from the older to the younger leaves in deficient plants. In chloroplast fragments of nitrogen-deficient and sulfur-deficient plants there exists a linear relationship between chlorophyll, protein sulfur, and protein nitrogen (78). Kylin (150) followed the uptake, assimilation, and distribution of sulfur (in part by the use of S^{35}) into three raw fractions during the germination process of wheat embryos. Sulfur deficiency manifested itself also in this case, as expected, in a marked increase of Kjeldahl nitrogen in the fraction containing the soluble nitrogen compounds, together with a lowering of the total nitrogen level. Šavloskij (243) has reported an assimilation of amino acid sulfur and other organic sulfur compounds from autolysates of bacteria and yeast by buckwheat embryos, and concludes that microorganisms of the rhizosphere have a special importance for sulfur uptake in higher plants. Shrift (253, 254) has found that a *Chlorella* strain assimilates methionine and also cysteine (not cystine) instead of sulfate; however, there appear to be differences in this regard between various algal types. Shrift's experiments on *Chlorella* also deal with the question of the toxicity and antimetabolic effect of selenium compounds. Selenate (253) in the presence of sulfate disturbs growth according to the scheme of competitive inhibition. This occurs partly in the absorption phase and partly in the realm of cell enzymes. Large quantities of sulfate can mitigate the effect of selenate; methionine also counteracts selenate toxicity, but not as a competitive antagonist. It is interesting to note the competitive inhibition of methionine (254) by the homologous selenium compound. Here occurs an adaptation which counteracts the toxic effects of selenomethionine. After a lag-phase, during which cell division is hindered and giant cells temporarily appear, the growth of the selenium culture (which contains normal methionine) approaches normality. The adaptation has been maintained over two passages of selenium-free cultures. This effect, which represents an interesting counterpart to the behavior of unicellular algae toward the ion pair $K^+ - Rb^+$ (215), has been interpreted as a modifying enzyme adaptation. A mutative adaptation, however, which has not been discussed, is more probable, according to the opinion of the present writer. Selenium produces, in contrast to Rb^+, no replacement effect, although selenium compounds, in addition to their competitive effect, are included to a slight extent in organic cell metabolism. The optical isomeres of methionine are not equivalent in these experiments. Preliminary experiments on the behavior of respiration and photosynthesis in sulfur deficiency and in the corresponding recovery experiment (cf. Table I) show that sulfur deficiency (like deficiencies of magnesium, iron, and nitrogen) causes disturbances which can only be corrected slowly. Sulfur deficiency interferes indirectly in the process of photosynthesis (212); the interference occurs apparently through the protein level and the chlorophyll content of the chloroplasts.

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Hitherto, the examinations of sulfur-deficient plants have not made evident any disturbances which would indicate a selective influence on structurally or biochemically effective S—S bridges or sulfhydryl groups. At present we are limited to the use of specific inhibitors. In this connection mention should be made of the observation of Lewin (157, 158) that cadmium chloride as an SH inhibitor specifically prevents the assimilation of silicon dioxide by diatoms, which is shown to be an aerobic process.

Iron.—A great deal of effort has again been made in recent years to trace chlorophyll deficiency and other disturbances appearing upon excessive treatment with various salts to interferences with iron metabolism. Such indirect iron-deficiency effects, particularly chloroses, have been described for treatment with cobalt (21, 117, 199, 280), nickel (117, 200, 280), copper (117, 259), zinc (259), manganese (21, 259), and cadmium (117), based on typical visual symptoms or on a response to subsequent application of iron compounds. Hewitt (117, 118), who deals with these problems most thoroughly, emphasizes above all the species-independent effect of copper on the availability of iron, whereas in cobalt, nickel, and manganese nutrition the effects are not so striking and unmistakably related to iron. The bivalence of the metals seems to play a part, but this is not true of a change of valence or of the redox potential of simple ion pairs. Iron chlorosis often is only the early phase of excess injury which later become specific. In combination the heavy metals often do not act additively, but may even detoxify each other. Among other problems, that of the equivalence of Co^{++} and Ni^{++} in their influence on the iron balance has been often discussed. Nickel-induced chlorosis is, according to Nicholas & Thomas (200), absolutely independent of iron. Also the best known effect of excess, namely, lime-induced chlorosis, requires still further analysis. Brown (44), assumes that plants with a highly developed iron-regulated enzyme system (especially catalase) are susceptible to lime-induced chlorosis, but are resistant to a copper deficit which exists in certain slightly acid "organic" soils. However, plants with many copper-containing enzyme (ascorbic acid oxidase) respond strongly to the latter condition.

Enzyme analyses in lime-induced chlorosis and in iron and copper deficiencies support these conjectures (46, 47). Iljin (132), on the other hand, has confirmed his view that Ca^{++}-chlorosis can in no case be explained by an iron deficiency *per se;* the amount of iron in chlorotic organs can even be supernormal. If calcium-sensitive plants are forced to grow under the same conditions as calcium-tolerant plants, such heavy disturbances arise that a decrease of chlorophyll may also develop without a direct relation to the mineral factors, e.g., in connection with a disturbance of protein metabolism. The phenomenon of lime-induced chlorosis, therefore, has to be viewed in relation to the over-all biochemical state of the plant. The iron level is, according to Hewitt & Bolle-Jones (121), also influenced by potassium supply. Potassium seems to increase the utilization of iron; iron, on the other hand, seems to inhibit the translocation of potassium. All above-mentioned results may, it is true, give an impulse to futher study, but they do not offer

new insights into the physiological role of iron itself. In iron-deficient plants, Bolle-Jones & Notton (39) observed a shift of the pigment relation, in that chlorophyll and carotene are more affected than xanthophylls.

Iron-deficient, chlorotic green algae have a lowered photosynthesis as is well-known. In the manometric recovery experiment (Table I) iron proves to belong to that group of elements, the absence of which causes a disturbance of the photosynthetic apparatus which is reparable only gradually in connection with new formation of the pigment (or with supplement of the protein deficit) (218).

The endogenous respiration of autotrophic cells does not respond markedly to moderate iron deficiency. We still have little information concerning the status and role of the iron accumulated, as is known, in the chloroplast. Of more recent results, mention may be made here of the final proof, as well as the spectroscopical characterization and chemical examination of cytochrome-*f* by Davenport & Hill (65, 124). The heme, which is particularly closely bound to protein, represents, however, only a relatively small portion of the plastide iron. This alone does not exclude the not yet clearly definable hypothesis that the heme has an importance in photosynthesis analogous to that of the cytochromes in aerobic respiration. Proof is still lacking of a general occurrence of cytochrome-*f* in green plants. Of great interest are reports from Boichenko & Baranov (38), according to which they were able to transfer a complex of iron with polyuronides from chloroplasts into aqueous solution; this complex is said to bind $C^{14}O_2$ in light and in the absence of chloroplasts, although much less than in their presence. Whether here a new approach to an artificial photosynthesis is possible, cannot yet be decided. Since Hill & Hartree (125) reported on hematin in plants, there do not appear to be any important new results in this sector concerning the function of these components. Egle & Munding (76) have contributed to the discussion of the function of hematins in bacterial nitrogen fixation; they were not able to demonstrate the hemoglobin of the leguminous root nodules in *Alnus*, *Hippophae*, and *Myrica*, but rather found cell hematins with other spectral properties in a relatively high quantity, so that it may be assumed that a direct or indirect function in nitrogen fixation can also be ascribed to these. New observations of cytochromes in roots [Lundegårdh (168)] and their cooperation in ion uptake will be reported on elsewhere.

Manganese.—In dealing with this element it is advisable to start with its effect on green algae, since, in this case, an examination of deficient cells and the application of recovery technique furnish a relatively clear picture (cf. Table I). In nutrient solutions without additional manganese there occurs an inhibition of growth, at first without any signs of chlorosis. In direct connection with this there is a lowering of photosynthesis, whereas respiration, nitrate reduction, and the oxidative assimilation of glucose in darkness remain at first completely normal. This is in contrast to all other hitherto examined cases of mineral deficiency. After addition of manganese, a complete return to normal photosynthetic activity occurred within a period

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of one or two hours. Similar behavior in *Chlorella* was described some time ago by Pirson (209), and has recently demonstrated in *Scenedesmus* by Arnon (11), so that there is hardly any doubt that this behavior may be considered typical at least for unicellular green algae. Inhibition of photosynthesis and recovery are demonstrable, in both strong and weak light, directly above the compensation point (218) as well as with approximately ⅓ light saturation (11). The limitation of manganese effect to photosynthesis is especially emphasized by the findings of Bergmann (32, 211), according to which *Chlorella* shows no inhibition of growth in heterotrophic culture with glucose despite absence of manganese. Dark cells of this type immediately show a marked and reversible inhibition of photosynthesis when exposed to light; the photosynthetic apparatus which is developed in darkness is thus manganese-defective, without any other demonstrable disturbance. An unexpected behavior was shown by algae cultivated in light with sugar but without manganese. The growth of these mixotrophic cells is noticeably inhibited in comparison with heterotrophic dark cells. In light, glucose consumption in cases of manganese deficiency ceases, but can be continued in darkness with a resumption of growth. Conversely, growth and glucose uptake cease in manganese-deficient heterotrophic dark cells upon exposure to moderate or strong light. Bergmann has concluded from this that the multiplication of mixotrophic cells does not result from the cooperation of heterotrophic and photosynthetic growth but rather that their glucose assimilation represents a light-dependent process, apparently a special case of photosynthesis, which, as such, is dependent upon manganese. Neish (191) has pointed out such a photosynthetic assimilation of sugar, which made possible growth in illuminated *Chlorella* cultures in the absence of CO_2 in a nitrogen atmosphere; this behavior recalls the anaerobic photosynthesis of *Rhodospirillum* [cf. (77)]. A similar experiment in which, under a steady current of nitrogen, Neish's findings were confirmed, indicated in turn that growth is dependent on the presence of manganese. Each application of the photosynthetic mechanism brings out the manganese requirement of algae. But at the same time it is not yet understood why the heterotrophic glucose assimilation mechanism is blocked, under sufficiently strong light, by manganese deficiency, and why manganese-deficient cells do not have an outlet to this mode of nutrition.

The Hill reaction of molecular oxygen, which was discovered by Mehler (176), and the similar peroxide formation in illuminated leaf extracts [Gerretsen (101)] are dependent on the addition of manganese. The same is true of the CO_2 reduction of illuminated plastides brought about by DPN in the malic enzyme system (9, 275, 286). The question must be left open whether in the above-mentioned chloroplast experiments a direct comparison with the experiments on algae may be made, since the chloroplasts came from leaves of normally fed plants; i.e., they are, at least *in vivo*, sufficiently supplied with manganese. Isolated chloroplasts do not accumulate manganese, but rather iron and copper [Whatley, Ordin, & Arnon (299)]; it is hard to

decide whether manganese was leached out during preparation or whether it occurs mainly outside the plastids in any case.

Enzymological experiences with the activating effect of manganese are so numerous and complicated [cf. (173)] that they cannot be used to clarify the special function of this element in photosynthesis, especially when it is assumed that only the photochemical part of the mechanism is affected. But, in any case, mention should be made of recent data on the function of manganese as a co-factor in oxidative phosphorylation [Lindberg & Ernster (161)], and of the already known relationship of manganese to enolase [Malmström (171)] and to glutamyl-transferase [Lajtha, Mela, & Waelsch (151)]. Since Hivon, Doty & Quackenbush (126) have found that the activity of ascorbic acid oxidase is not reduced in soy beans and green beans showing clear manganese-deficiency symptoms, they consider the relation, which has sometimes been mentioned, of manganese to this copper-containing enzyme to be disproved. Brown & Steinberg (47) have, on the other hand, found that in manganese-deficient tobacco the ascorbic acid oxidase is diminished; however, the enzyme is much more strongly affected by copper deficiency. A sharp rise in peroxidase activity in manganese-deficient plants is noticeable. At the onset of manganese deficiency the catalase activity in *Chlorella* remains unchanged, in spite of reversible inhibition of photosynthesis [Bergmann (32)].

Studies have been made of manganese effect upon higher plants which suggest a direct comparison with the experiments on algae. These experiments concern mainly the addition of manganese to manganese-deficient plants, i.e., a kind of recovery technique which admittedly involves long-term effects. It may be anticipated that a comparison of results here will be disappointing to those who hope to find a direct similarity of behavior between algae and higher plants. In 1942 Reuther & Burrows (228) followed the effect of a strong addition of manganese (immersion in a 1 per cent $MnSO_4$ solution) upon photosynthesis in manganese-deficient young tung trees under natural conditions. A moderate increase in photosynthesis was observed, but only in the youngest leaves. Portsmouth (222) later found, with the help of a modified half-leaf method, that, after injection of $MnSO_4$ (0.025 per cent) in manganese-deficient potato leaves, there was an inhibition of apparent photosynthesis, which was perhaps caused, or at least partially determined, by a simultaneous increase in respiration. Gerretsen (100) has observed an inhibition of photosynthesis, without any considerable decrease in chlorophyll, in oats, the classical object for examination of manganese deficiency; however, he did not follow this up with recovery experiments. From a simultaneous study of visual deficiency symptoms he concludes that there is a special influence of manganese on photosynthesis. Most recently Ruck & Bolas (240) have performed experiments on manganese deficiency and recovery in rooted leaves of potato, which, due to the absence of various complications, have been found to be good experimental objects [Gregory & Samantarai (107)]. Net assimilation is inhibited by manganese deficiency,

without there being any direct connection with the chlorophyll content, which is likewise decreased; apparently there is also a decrease in respiration. Addition of manganese through the roots produced no change in net assimilation or respiration.

In general, these experiments indicate, in spite of certain contradictory evidence, an inhibition of photosynthesis, but still this effect is not as clear and well-separable as it is in algae. It is hard to judge the reasons for this difference. Perhaps the first stage of manganese deficiency, which can be so clearly ascertained in algae, passes by too quickly and gives way to a deep-lying disturbance such as also appears in algae during the late stage of deficiency. Manganese deficiency possibly has various consequences, depending upon whether it is produced by removing the element from leaves which are already formed, or from young leaves. The total disturbance picture, as found in the literature of manganese deficiency in higher plants, is admittedly so complicated that the possibility of a behavior pattern completely different from that of algae must be further examined.

The cooperation of manganese and iron within a redox system in respiration or photosynthetic metabolism, which has often been mentioned but never satisfactorily demonstrated, is no longer of such primary importance since the last reviews of Twyman (278), Hewitt (116), and Mulder & Gerretsen (186). The independent effects of these elements at both deficiency and excess levels have received greater emphasis. Löhnis (163, 164, 165) has continued her extensive examinations of manganese-excess effects in numerous culture plants. A resistance to injury is often traceable to a reduced uptake of manganese, but in some cases also to plasmatic factors. Little or no liming can, even without addition of manganese, cause spontaneous chlorosis and other damage attributable to manganese excess; this has been especially pronounced in the sensitive bean plant. Symptoms of excess in field experiments differ from those of iron excess, but this is not the case in solution cultures. There is a clear uptake antagonism between manganese and magnesium (165), so that an addition of magnesium prevents the appearance of injury symptoms in a medium with Mn-excess. Owen & Massey (206) have observed, after liming or at high pH values, strong chloroses in roses resulting from manganese deficiency and not from iron deficiency. The effect of excessive manganese can be weakened, as is also the case with excesses of molybdenum and vanadium, by large doses of iron; conversely, a large quantity of manganese, molybdenum, and vanadium reduces iron content in soy beans, peas, and flax [Warington (293, 294)]. According to Bakr-Ahmed & Twyman (21) iron in tomato plants is bound by manganese excess (5 to 10 p.p.m.) and even more by cobalt addition (from 0.1 p.p.m. upward). In addition to their effect on iron metabolism, cobalt and manganese exert other injurious influences. Cobalt and manganese can detoxify each other; here cobalt is the stronger component. Hewitt (118) has observed that an excess of Cu^{++} Cd^{++}, and Co^{++} cause iron-deficiency chlorosis more easily than does an application of Mn^{++}. According to the visual symptoms, Ni^{++} and Zn^{++}

appear to produce a manganese-deficiency chlorosis. As yet the situation here is quite confusing, especially since the various species behave differently. This is strongly emphasized by comparing the toxicity effects of numerous micronutrient elements which have been described by Hunter & Vergnano (131). In all hitherto described cases of excess effects there has, however, not yet been a satisfactory causal analysis from a physiological point of view. This is also true of the detoxifying effect of manganese addition (10^{-3} *M*) in streptomycin-inhibited growth of *Avena* coleoptile sections which can also be brought about by a double quantity of calcium [Rosen (239)]. It may be mentioned in passing that Cooil (57) has succeeded in enhancing the growth of coleoptile sections with potassium salts and even more so by adding manganese simultaneously. According to the latest data from Leach *et al.* (155) there can no longer be any doubt that the well-known grey speck disease in oats is due entirely to manganese deficiency; it is striking that in this case a large quantity of calcium is able to delay the formation of deficiency symptoms.

Molybdenum.—The question of the significance of molybdenum and its part in plant metabolism offers a prime example of the many-sided problems which face the physiologist in studying mineral nutrition. This particular field has been rapidly developed from both the practical and the theoretical side [cf. (115, 183)]. However, the opinions of the various researchers in regard to the function of this micronutrient are still controversial and in part quite hypothetical.

As is well known, molybdenum was first considered to be a special catalyst for biological nitrogen fixation, and furthermore a co-factor in nitrate reduction in fungi and higher plants. The demonstration of a minute molybdenum requirement in higher plants, even in cases of exclusive ammonium nutrition, was still considered doubtful not long ago, but today it seems to be proved (2, 123). Therefore, the question arises whether there are in plants several systems containing molybdenum-dependent enzymes or structures with various requirements, as has evidently been worked out in the case of calcium (51, 52). On the other hand, it would be more satisfactory if a single reaction center could be discovered at which molybdenum specifically acts, from which there would easily result an indirect control of several peripheral reactions. In this case there could also appear associated reactions with varying molybdenum requirements, depending upon the degree of chemical or energetic needs in regard to the primary reaction.

Experimental guidelines for both alternatives have been laid down by studies on various objects, i.e., blue-green algae and green plants, especially Leguminosae. Wolfe (306) has carried out manometric measurements and analyses of nitrogen and carbohydrate fractions through careful comparisons, together with culture experiments, on *Anabaena cylindrica;* there was found a cessation of protein synthesis in nitrate-supplied cells with molybdenum deficiency, but yet there was a clear reduction of nitrate to the level of ammonium or amide. The blockage, caused by molybdenum deficiency, thus

lies evidently within the range of carbohydrate metabolism. Nitrate reduction can be increased by adding fumarate (also succinate and citrate, but hardly by glucose or acetate), just as by adding molybdenum, but the main effect here is a normalization of protein synthesis. Thus, molybdenum appears to control the supply of hydrogen donors or energy carriers which are necessary for protein synthesis. Fogg & Wolfe (90) assume, with support from Wood (306a), that molybdenum, at least in this special function, regulates the assimilation of respiratory energy in endergonic processes through the ADP/ATP system, as well as the central supply of hydrogen donors; they also support this view by experience with the inhibition of hydrolysis of phosphate esters by molybdenum traces [most recently, cf. (298)]. According to this the increased respiration in molybdenum-deficient algae, which has also been observed in *Chlorella* by Loneragan & Arnon (167), would constitute an "idling" comparable to the reversible respiratory increase in potassium deficiency (218). The nitrogen fixation in blue algae could be fitted into this picture as a process only indirectly dependent upon molybdenum. But, in any case, this supposition needs further experimental support and can as yet serve only as a working hypothesis.

On the other hand, it appears certain that nitrate reductase, which could be isolated from *Neurospora* and which controls the first phase of nitrate reduction (nitrate→nitrite), consists of a flavine-adenine-dinucleotide with molybdenum as activator (196, 197, 198). Evans (82) has also attempted to connect the nitrogen fixation directly with this system; i.e., his view is almost the direct opposite of Fogg & Wolfe's. Since the root nodules of Leguminosae show several times the molybdenum content of the roots themselves and since the nodule bacteria are particularly rich in nitrate reductase, it is conceivable that molybdenum takes part, through nitrate reductase, in one step of nitrogen fixation; this then would have to be an oxidative one. The copper sensitivity of nitrogen fixation, which has been shown in pot experiments, fits in well with the inactivation of nitrate reductase from nodules through copper ions; this can be understood as an antagonistic effect in regard to molybdenum. For further discussion it is particularly important to become familiar with the nitrate reductases of blue algae, green algae, and higher plants. It is not yet known whether these also contain molybdenum or whether they function in a basically different way, as appears to be the case in the nitrate reductase of *Escherichia* (242).

Walker (288) and Arnon *et al.* (11, 14, 167) have also demonstrated a molybdenum requirement in *Chlorella* and *Scenedesmus*. For the time being this is known to be true only in a nitrate medium; a molybdenum requirement in urea supply has not yet been ascertained (288). Arnon (11) reports a marked reduction in chlorophyll in a nitrate-containing medium as an early deficiency symptom which can be corrected by the addition of molybdate. Photosynthesis appears to be only indirectly affected, through the interference with chlorophyll synthesis by molybdenum deficiency.

New observations of molybdenum requirement and uptake, as well as

of deficiency symptoms in higher plants, especially for tobacco, are available [Steinberg (265, 266)]; the activity of ascorbic acid oxidase is lowered (47), protein is reduced, and, as in many other cases of molybdenum and other deficiencies, nitrate is accumulated. Cotton does not take up less molybdenum from nutrient solutions than Leguminosae do [Joham (135).] For these latter, Vinogradova (283) has found that the requirement varies in the taxonomic sequence Papilionoideae, Mimosoideae > Sophoreae > Caesalpinioideae. Hewitt *et al.* have continued their comprehensive culture experiments on the molybdenum problem. The typical visual deficiency symptoms, especially chlorosis, appear clearly only if nitrate is supplied. In other nitrogen sources (nitrite, ammonium salt, urea, glutamic acid) the molybdenum requirement manifests itself mainly only in the reduced size of the plants. Whether the differences here are fundamental or only quantitative is not yet clear. Ascorbic acid and dehydroascorbic acid are independent of the nitrogen source, but are reduced in somewhat various degrees in cases of molybdenum deficiency (123). The reductive effect of triphenyl-tetrazolium chloride is lowered in the tissue of typical molybdenum-deficient plants (120). This symptom is not actually specific; Brown (45) has found a complete cessation of the TTC reaction in plants with a clear copper deficiency, parallel to the lessening of the activity of ascorbic acid oxidase. Addition of molybdenum, in combination with other heavy metals, accentuates most of the artificial excess chloroses produced by the latter. The similarity between the effects of molybdenum and copper in this case does not agree with enzymological findings, according to which molybdenum enzymes are inhibited by copper (82).

Molybdenum deficiency is more widespread in soils than was formerly assumed (40, 136). Molybdenum deficiency in serpentine soils is especially noticeable in cases of liming; Johnson, Pearson & Stout (136) have observed deficiency symptoms in many crop plants in this mileu. Bortels & Wetter (40) have pointed out the shortcomings of the *Aspergillus* test as a means of determining the molybdenum requirements of soils. Seed reserve plays an especially important part in molybdenum supply (122, 174). Hewitt (115) and Virtanen (285) have dealt with the general methodological importance of seed reserves for experiments with micro-nutrients and their successive diminution through culture sequences.

Since the occurrence of the flavine enzyme xanthine oxydase in green plants has not yet been clarified, and in any case would not seem to be particularly important here, it may be mentioned in passing that De Renzo *et al.* (227) have produced further documentation for the function of molybdenum as a co-factor in this enzyme [cf. (173)]. All previous experiences with molybdenum indicate that this element takes part in electron transport, but direct proof of this has yet to be offered.

Vanadium.—Molybdenum and vanadium have heretofore often been treated together, due to reports indicating that vanadium could replace molybdenum in Leguminosae and free nitrogen-fixing bacteria. But today

this view can no longer be held, now that the specificity of vanadium in plant metabolism has been proved. There are data on an enhancement of barley by an addition of vanadium without molybdenum (97), and also on a specific effect of vanadium in the range of a more excessive dosage. Warington (293) has found that a chlorosis in soy beans, flax, and oats, due to manganese excess, is diminished by vanadium, but increased by molybdenum. Excessive vanadium alone produces damage without initial chlorosis. Iron-deficient plants have an increased sensitivity to the addition of vanadium. A satisfactory explanation of the effects of manganese, molybdenum, and vanadium in various combinations, which have been further examined (294), is scarcely possible.

The situation in regard to the function of vanadium in algae is much clearer. Here vanadium was recently recognized by Arnon & Wessel (16) as a necessary micro-nutrient; i.e., it cannot be replaced by molybdenum, or vice versa. An enhancement of growth in *Scenedesmus obliquus* by vanadium is observable in a much greater range of concentration than is the enhancement by molybdenum (up to 100 μg./l versus 0.1 μg./l). Vanadium deficiency does not produce such a marked reduction in chlorophyll content as does molybdenum deficiency. In strong light, photosynthetic oxygen production in vanadium-deficient cells is inhibited, but can be reactivated by vanadium if sufficient time is allowed for recovery. This effect differs from manganese deficiency in which the rate of photosynthesis as measured by oxygen evolution is depressed in both weak and strong light, but is rapidly restored upon the addition of manganese. Thus, vanadium appears to affect photosynthesis, probably as far as the dark reactions are concerned.

At the present time, it is still not known in which form or complex vanadium is present in living green cells, where, according to Bertrand's (33, 34) older data, it is quite widespread. Yet it may be pointed out that the vanadium pigment (Haemovanadine) which was found in the blood cells of Ascidiae (Tunicatae) in 1911 by Henze (113) is just now being exhaustively studied in several laboratories [Bielig *et al.* (35, 36); Boeri & Ehrenberg (37)]. Optical data show that the vanadium occurs in a trivalent form; it yields a salt-like compound with coordinatively bound sulfuric acid on protein (molecular weight of the chromoproteide approximately 24400, with about 24 atoms of vanadium per molecule). The vanadocytes accumulate the vanadium from sea water up to ca. $3 \cdot 10^6$ times (36).

Copper.—The hypotheses on a cooperation of copper-containing systems in photosynthesis [cf. Arnon (6)], which have been considered particularly in regard to the selective localization of copper in chloroplasts (191, 299) have not been directly refuted but pushed into the background by other results of photosynthesis research; yet the problem is by no means solved. The often-discussed question of the importance of copper enzymes in terminal oxidation in green plants also requires a detailed experimental stimulus. It has been proven several times (45, 46, 47) that a decrease of copper may affect the copper-containing enzymes in the plant. It is remarkable that,

according to the findings of Gilbert (103) which have been confirmed by further experiments, in clearly copper-defective tung trees protein and chlorophyll are not reduced, but rather increased in comparison with normal plants. Thus, copper deficiency seems to occupy a special position. The question is open, to what extent this behavior represents a kind of preservation by blocked proteolysis, and whether, in this case, it is copper deficiency itself, or the relative surplus of a factor normally superseded by copper, which is effective. Conversely, a high proportion of protein can inactivate the cupric ions, probably as a result of the formation of stable proteids. For this reason, abundant nitrogen supply favors the increase of copper-deficiency symptoms.

Large-celled algae such as *Hydrodictyon* often must be protected against the toxic effect of small quantities of heavy metal (especially copper) by application of soil extract [or other chelating agents (189)]. Therefore, it is striking that, according to Walker (288), in *Chlorella* copper belongs to the essential micronutrients; the details of its function are not known in this case. An apparently mutative adaptation to higher, normally injurious copper doses has been observed in *Ankistrodesmus* (142, 215). In this connection more detailed experiments on copper adaptation of yeasts may be mentioned (182, 308); as a cause of this adaptation not only a spontaneous mutation, but also a mutagenous influence of copper has to be considered. A preparation of ribonucleic acid from the copper-adapted yeast cells is reported to induce copper resistance in normal cells. Experiments on the effects of copper excess in higher plants have been partly touched upon in the previous sections and need no further mention here.

Zinc.—A marked and fairly uniform chlorosis, together with the well-known "little leaf" symptom, has recently been described again as a striking sign of zinc deficiency in cotton (48), *Phaseolus* (282), and corn (281). The leaves of the deficient plants show a greater lack of zinc than do the shoot parts. The tips of the shoots may at first retain a normal zinc content (281, 282). In *Chlorella* the chlorophyll is also very strongly affected by zinc deficiency, and is selectively restored by addition of zinc (203, 261). Apparently, there is an early but robust interference in protein synthesis which also directly involves the protein fraction of enzymes, including that of zinc-activated carbon anhydrase [Wood & Sibly (307)]. In view of this situation there must be expected an immediate appearance of numerous secondary deficiency symptoms, which makes a physiological analysis of zinc effect quite difficult. Therefore, there are still divergent opinions on the part played by zinc. Vöchting (287) examined the shifting of zinc within the grain and embryo of corn during germination; here the element is obtained from the embryo with scutellum. *Aspergillus* grown with corn in mixed culture may successfully compete with the latter for the zinc in the nutrient solution. Bergh (30, 31) has experimented with radioactive zinc (Zn^{65}) on the uptake of zinc from soil. Moreover, he found (29) that of several different crop plants, the tomato was remarkably resistant to zinc excess; it responded even to

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high dosages with an increase in production. Shear (252) has reported an increased resistance to cold in tung trees as a result of excess zinc doses; this effect appeared only when large quantities of potassium were added at the same time, and as yet cannot be explained on a physiological basis. In culture experiments with plants from natural calamine soil (*Silene inflata*) Baumeister (26) has observed an apparently inherited resistance to zinc. This manifests itself in increased photosynthesis and respiration, if the calamine plants in a zinc-excess medium are compared with normal controls. Furthermore, a moderate enhancement of photosynthesis related to leaf area can be noted after raising the zinc level. The zinc-type plants also differ from the normal type in regard to constant morphological characteristics (prostrate growing, small leaf area).

Cobalt.—A report should be made here on cyanocobalamine (vitamin B_{12}) and hydroxocobalamine (vitamins B_{12a} and B_{12b}), their biogenesis and function, even if only of organisms containing chlorophyll, in a more detailed way than is possible in this review. In regard to this special field, which, at least at present, lies somewhat outside the field of nutritional physiology of green plants, the reader may be referred to a special review of Darken (64). Here it can only be stressed that, in view of the hitherto available statements on an occurrence of cobalamines in higher plants, there is a likelihood of microbe origin, so that these statements have to be accepted with reservation. Details on these vitamins as lately gained, may further be gathered from the recently published comprehensive work on vitamins edited by Sebrell & Harris (251). Ericson & Lewis (81), as well as Lewin (159), have reported on the vitamin B_{12} factors occurring in marine algae (*Stichococcus*). *Chrysomonadales* (23) seem to respond only to vitamin B_{12} itself and not to related compounds. A further experimental report exists on *Euglenae* (237).

In the range of excess (10^{-5} to 10^{-4} *M*), Miller (179) has found, after adding Co^{++} ions, a strong enhancement of elongation in etiolated pea-stem sections, but only, however, in the presence of IAA and sugars (especially sucrose). The assumption that the cobalt ions act specifically on the properties of the cell membrane may be related to similar views of Burström (51, 52) on the effect of Ca^{++}. Thimann (274) has affirmed the effect of cobalt in pea stems, but not, however, in the *Avena* coleoptile. Also the expansion of slices of etiolated bean leaves is favored by Co^{++} [as is also the case with nickel (178, 180)]. Cobalt and illumination acts in a parallel manner, and, if jointly given, in an additive way. It can be assumed that the heavy metal, which in these doses is unfamiliar to the cell, works indirectly via an unblocking, e.g., by making Ca^{++} available. That a surplus of Co^{++} interferes in iron metabolism, but also may cause certain symptoms of its own, has been mentioned already in the section on iron. Ballentine (22) has shown (so far in *Neurospora*) that cobalt in the course of growth and metabolism is incorporated to a slight extent in stable protein complexes. Growth tests with graduated cobalt and iron doses, after being examined with the aid of

variance analysis, make it appear probable that cobalt functions as an independent complex former and is not substituted for iron as a result of a "metabolic mistake." Such efforts naturally will not become directly interesting for the physiologist until a general essentiality of cobalt has been proved.

Boron.—Although, as has been stated in a review by Baumeister (25), the physiologists are directing their interest more and more to the effects of boron, this micronutrient still remains their particular problem child. This situation will be unchanged as long as the primary effects of boron continue to be sought, as they perhaps must be, in the little-known field of "plasma colloids," and as long as no higher differentiated reaction centers can be examined.

A short remark of Eyster (84), to the effect that boron deficiency in *Nostoc muscorum* appears with a marked loss of pigment together with a slight inhibition of growth, seems so far to be the only explicit reference to an essentiality of boron in green micro-organisms. Corresponding statements concerning heterotrophic forms are also very scanty and not particularly concise. In contrast to cobalt, however, the essentiality of boron is, as is well-known, clearly observable in higher plants; it may therefore be regarded as a micronutrient more on a tissue than on a cellular basis.

A striking effect here is the acceleration in the uptake of tracer sucrose in the laminae of tomato leaves, and its distribution in the plant after addition of boric acid (10 p.p.m.), as has been observed by Gauch & Dugger (96). By favoring the introduction of sugar, boron also enhances tissue respiration. The borate ions are reported to form, with sugars, ionizable complexes which permeate more easily than do nonionized sugar molecules. In general, boron deficiency is assumed to manifest itself often by difficulties in carbohydrate transport. However, Ashworth (18) has rather placed an influence of boron upon pectic substances. Stunting of internodia, inhibition of the development of leaf area, and enhancement of lateral buds are reported by Askew & Monk (19) as a deficiency symptom in hops.

A detailed anatomical diagnosis of deficient plants of *Hibiscus cannabinus* by Hirai (130) generally indicates serious disturbances in protoplasm as well as in the cell wall (poor development of bast fibers, wound gummosis). Further observations of the symptoms of boron deficiency (85, 170) do not offer direct explanation of the physiology of boron effects even in connection with analyses of mineral contents (260). Pope & Munger (221) have found in celery great differences in variety in regard to sensitivity toward omission of boron; this seems to be a mono-hybrid-inherited property [cf. (220)].

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XUM

BIOCHEMICAL ASPECTS OF PHOTOSYNTHESIS[1,2]

By Wolf Vishniac[3]

Department of Microbiology, Yale University, New Haven, Connecticut

The past year has seen two major developments which are contributing to our understanding of photosynthesis. First, ribulose diphosphate has been established as the donor of the active two-carbon fragment which accepts carbon dioxide in photosynthesis. Secondly, the discovery of a variety of cytochromes promises to elucidate some of the reactions associated with oxygen evolution. This review emphasizes the experiments that bear on these two aspects of photosynthesis.

The chemical aspects of photosynthesis have recently been reviewed by Holzer (1). The photosynthetic bacteria have been discussed in a review by van Niel (1a). For more general information and for references to earlier work the reader should consult the excellent reviews of Lumry, Spikes, & Eyring (2) and Brown & Frenkel (3), as well as earlier reviews of this series and the monograph of Rabinowitch (4).

The Reduction of Carbon Dioxide

Fixation of carbon dioxide.—Experiments in which algae were permitted to photosynthesize for short periods in the presence of $C^{14}O_2$ established phosphoglyceric acid as the "first product" of photosynthesis [Benson *et al.* (5)]. The term "first product" here means that the earliest stable, tangible organic compound to contain a carbon atom derived from cabon dioxide is phosphoglyceric acid. These findings have been confirmed by Gaffron and his collaborators (6). The radioactivity administered as $C^{14}O_2$ appears first in the carboxyl carbon of phosphoglyceric acid, later also in carbons 2 and 3 which become equally labelled. Although Calvin *et al.* (7) first believed that the first product was 2-phospho-D-glyceric acid, it now appears, especially from the work of Quayle *et al.* (8) and Weissbach *et al.* (9), that car-

[1] The literature survey for this review was concluded in December, 1954.

[2] The following abbreviations will be used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DAP, dihydroxyacetone phosphate; DNP, 2,4-dinitrophenol; DPN, diphosphopyridine nucleotide; DPNH-reduced DPN; E-4-P, erythrose-4-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P_2, fructose-1,6-diphosphate; G-6-P, glucose-6-phosphate; Gl-3-P, glyceraldehyde-3, phosphate; P_i, orthophosphate; 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; 1,3-P_2GA, 1,3–diphosphoglyceric acid; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; Ru-1,5-P_2, ribulose-1,5-diphosphate; S-7-P, sedoheptulose-7-phosphate; S-1,7-P_2, sedoheptulose-1,7-diphosphate; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN.

[3] The reviewer is indebted to Dr. B. L. Horecker for a discussion of carbohydrate formation, but assumes sole responsibility for speculative views.

bon dioxide is first incorporated in 3-PGA.[2] Since CO_2 is first transformed into the carboxyl group of 3-PGA, a search was made for the two-carbon compound which upon carboxylation would become 3-PGA. Fager (10, 11, 12) examined several such compounds and came to the conclusion that vinyl phosphate, phosphoglycolic acid, and phosphoglycolaldehyde were not intermediates in the formation of phosphoglyceric acid. Bassham *et al.* (13), on the basis of their isotope studies, similarly came to the conclusion that glycolic acid was not closely related to the carbon dioxide acceptor. The search for the two-carbon compound active in phosphoglyceric acid formation parallels the study of that aspect of carbohydrate metabolism which culminated in the discovery of the transketolase reaction [Racker *et al.* (14, 15) and Horecker & Smyrniotis (16)]. The interconversions of carbohydrates have recently been reviewed by Racker (17). In the splitting of pentose phosphate which precedes the formation of sedoheptulose phosphate no free two-carbon compound arises; instead, a suitable acceptor, here an aldehyde, must be present to which two carbons are transferred. Similarly, it was suggested by Calvin & Massini (18) that no free two-carbon compound might occur to accept carbon dioxide in photosynthesis, but that a pentose might serve as donor of the two-carbon unit. Ru-1,5-P_2[2], which Benson (19) found to be rapidly labeled during photosynthesis with $C^{14}O_2$, was found to serve as the two-carbon donor in the formation of 3-PGA. The evidence for the role of Ru-1,5-P_2 is based on isotope distribution data [Bassham *et al.* (13) and Zweifler (20)] and on the occurrence of the enzymatic reaction

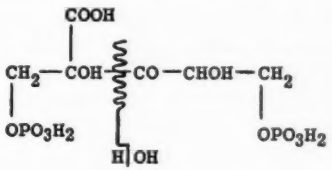
$$\text{Ru-1,5-P}_2 + CO_2 \rightarrow 2\ \text{3-PGA} \qquad 1.$$

which has been observed by Quayle *et al.* (8) in *Chlorella* extracts and by Weissbach *et al.* (9) in preparations from spinach. Reaction 1, unlike the TPN[2]-linked CO_2-fixing reactions catalyzed by malic enzyme, isocitric dehydrogenase, and phosphogluconic dehydrogenase, does not require an additional hydrogen donor. It, therefore, invites comparison with the nonreducing CO_2-fixing reactions: the carboxylation of phosphoenolpyruvate which was observed by Utter & Kurahashi (21) and by Bandurski & Greiner (22) (reaction 2), and the carboxylation of propionyl-CoA which was found by Whiteley (23) (reaction 3).

$$\text{Phosphoenolpyruvic acid} + \text{IDP} + CO_2 \rightarrow \text{Oxalacetic acid} + \text{ITP} \qquad 2.$$

$$\text{Propionyl-CoA} + CO_2 \rightarrow \text{Succinyl-CoA} \qquad 3.$$

However, in both these reactions carbon dioxide is attached to a methyl group at the end of a carbon chain, whereas in reaction 1 a C-C bond is formed between carbon dioxide and carbon 2 of ribulose. The mechanism of reaction 1 can be interpreted in several ways. Bassham *et al.* (13) suggest the formation of a six-carbon intermediate, possibly attached to the enzyme, which presumably would be a β-keto acid of the structure



The indicated split between carbon 2 and carbon 3 would then give rise to two molecules of 3-PGA. An alternative interpretation would be to regard reaction 1 as catalyzed by a C_2 transfer enzyme for which carbon dioxide serves as the specific acceptor molecule. The reaction could then be formulated as follows:

$$\text{Ru-1,5-P}_2 + \text{enzyme} \rightarrow \text{3-PGA} + \text{"active C}_2\text{"-enzyme} \quad 4.$$

$$\text{"active C}_2\text{"-enzyme} + \text{CO}_2 \rightarrow \text{3-PGA} + \text{enzyme} \quad 5.$$

The sum of reactions 4 and 5 is reaction 1. The partial reactions, if they are reversible, could be verified by isotope exchange experiments. The incubation of the enzyme with Ru-1,5-P_2 and radioactive 3-PGA should, in the absence of carbon dioxide, give rise to radioactive Ru-1,5-P_2. Similarly, a mixture containing the enzyme, 3-PGA, and radioactive carbon dioxide should lead to the incorporation of radioactivity in 3-PGA. The CO_2 fixation with Ru-1,5-P_2 was presumably the reaction which Fager (12) observed in spinach particles which formed 3-PGA. It is therefore surprising that he failed to stimulate the reaction with R-5-P and ATP.[2]

Formation of ribulose diphosphate and its precursors.—Weissbach *et al.* (24) have found in spinach preparations a ribulokinase which carries out the reaction

$$\text{Ru-5-P} + \text{ATP} \xrightarrow{\text{Mg}^{++}} \text{Ru-1,5-P}_2 + \text{ADP} \quad 6.$$

From the isotope distribution data reviewed by Bassham *et al.* (13) it appears that ribulose is labeled in carbons 1, 2, and, most heavily, 3. While no five carbon fragment of either the six or seven carbon sugars contains radioisotope in similar distribution, the labeling of ribulose may be accounted for by the transketolase reaction in which S-7-P and Gl-3-P give rise to R-5-P and Ru-5-P.[2] Since the S-7-P is labeled in carbons 3, 4, and 5, and the Gl-3-P arising from 3-PGA in carbon 1, the R-5-P is labeled in carbons 1, 2, and 3, and the Ru-5-P in carbon 3 (reaction 7):

$$\underset{\text{S-7-P}}{\text{C—C:O—C*—C*—C*—C—C—P}} + \underset{\text{Gl-3-P}}{\text{C*—C—C—P}} \rightarrow \underset{\text{R-5-P}}{\text{C*—C*—C*—C—C—P}} + \underset{\text{Ru-5-P}}{\text{C—C:O—C*—C—C—P}} \quad 7.$$

The isomerase reaction which converts R-5-P to Ru-5-P leads to an "average" labeling of Ru-5-P in accordance with the observed values. The labeling

of S-7-P in the 3, 4, and 5 positions may be accounted for in two ways. Either the S-7-P arises by the aldol condensation of E-4-P, labeled in carbons 1 and 2, with 1-labeled DAP to give S-1,7-P_2 (reaction 8) which is then hydrolyzed to S-7-P; or E-4-P undergoes a transaldolase reaction with F-6-P which is labeled in carbons 3 and 4 (reaction 9).[2]

$$\underset{\text{DAP}}{\text{P—C—C:O—C*}} + \underset{\text{E-4-P}}{\text{C*—C*—C—C—P}} \rightarrow \underset{\text{S-1,7-P}_2}{\text{P—C—C:O—C*—C*—C*—C—C—P}} \qquad 8.$$

$$\underset{\text{F-6-P}}{\text{C—C:O—C*—C*—C—C—P}} + \underset{\text{E-4-P}}{\text{C*—C*—C—C—P}}$$

$$\rightarrow \underset{\text{S-7-P}}{\text{C—C:O—C*—C*—C*—C—C—P}} + \underset{\text{Gl-3-P}}{\text{C*—C—C—P}} \qquad 9.$$

The DAP would arise by isomerization of the labeled Gl-3-P. The F-6-P could arise via F-1,6-P_2[2] from the aldolase condensation of the two triosephosphates. It would therefore be equally labeled in carbons 3 and 4. The E-4-P (labeled 1 and 2) can arise by a transketolase reaction between F-6-P (labeled 3 and 4) and Gl-3-P (labeled 1) which would yield E-4-P and Ru-5-P (labeled 3) (reaction 10).

$$\underset{\text{F-6-P}}{\text{C—C:O—C*—C*—C—C—P}} + \underset{\text{Gl-3-P}}{\text{C*—C—C—P}}$$

$$\rightarrow \underset{\text{E-4-P}}{\text{C*—C*—C—C—P}} + \underset{\text{Ru-5-P}}{\text{C—C:O—C*—C—C—P}} \qquad 10.$$

The formation of carbohydrates in photosynthesis.[3]—The isotope data accumulated in Calvin's laboratory [Bassham *et al.* (13)] combined with present knowledge of the enzymatic interconversions of carbohydrate phosphates [reviewed by Racker (17)] permit the sketching of a scheme which describes the incorporation of carbon dioxide into carbohydrates during photosynthesis. One such scheme, in part speculative, is presented in Figure 1. This scheme is based largely on the one presented by Bassham *et al.* (13) and differs mainly in the mode of formation of S-7-P. Bassham *et al.* (13) suggest an aldolase reaction between Gl-3-P and E-4-P to give S-1,7-P_2 which then undergoes hydrolysis. A S-1,7-P_2-phosphatase has however not yet been found. S-7-P is here thought to arise by a transaldolase reaction between E-4-P and F-6-P as discussed below.

The sequence of events outlined in Figure 1 leads to a net formation of Gl-3-P. Carbon dioxide is fixed by reaction with Ru-1,5-P_2 [reaction (a) in Figure 1] giving rise to two molecules of 3-PGA [Quayle *et al.* (8) and Weissbach *et al.* (9)]. In the presence of $C^{14}O_2$ the carboxyl group of 3-PGA would appear labeled. The 3-PGA so formed is transformed to 1,3-P_2GA[2] by reaction with ATP and phosphoglyceric acid kinase and reduced to Gl-3-P by triosephosphate dehydrogenase (reaction b, Fig. 1). It is in this reduction that hydrogen derived from water is transferred to carbon derived from carbon dioxide. The presence of phosphoglyceric acid kinase in green leaves has been established by Axelrod & Bandurski (25), while Gibbs (26) and

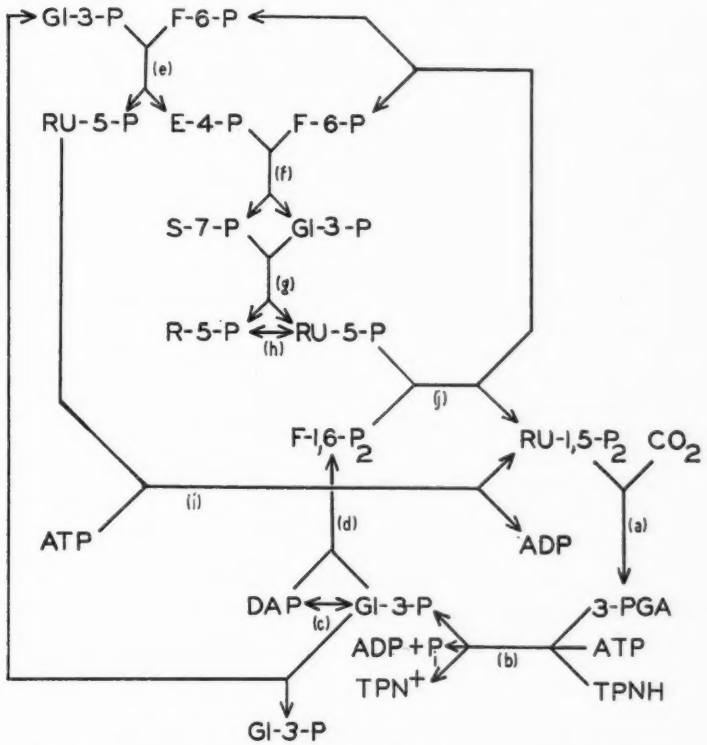


FIG. 1. Carbon dioxide fixation in photosynthesis. For explanation see text.

Arnon (27) have found a TPN-linked triosephosphate dehydrogenase. The fixation of three molecules of CO_2 in reaction (a) would thus lead to the formation of six molecules of Gl-3-P labeled in carbon 1. One molecule of Gl-3-P may be considered the net product. Two molecules of Gl-3-P isomerize to DAP (reaction c) and, with two more molecules of Gl-3-P, undergo the aldolase reaction to form two molecules of F-1,6-P_2 (reaction d) which is thus labeled in carbons 3 and 4. The occurrence of triosephosphate isomerase in green leaves has been mentioned by Axelrod *et al.* (28), and aldolase has been found in green leaves by Tewfik & Stumpf (29).

The sixth molecule of Gl-3-P acts as the acceptor in a transketolase reaction with F-6-P_i (reaction e) to give rise to Ru-5-P and E-4-P. The occurrence of transketolase in green leaves has been shown by Axelrod *et al.*

(28), Horecker *et al.* (30), and by Gibbs & Horecker (31). The Gl-3-P is labeled in the aldehyde carbon and F-6-P, since it arises from F-1,6-P_2 formed in reaction (d), is labeled in carbons 3 and 4. Reaction (e) therefore leads to Ru-5-P labeled in carbon 3 and E-4-P labeled in carbons 1 and 2. Ru-5-P can undergo the ribulokinase reaction of Weissbach *et al.* (24) to make another molecule of Ru-1,5-P_2 (reaction i). E-4-P may serve as the acceptor in a transaldolase reaction with another molecule of F-6-P (reaction f) yielding S-7-P and Gl-3-P. The radioactivity of carbon 3 of F-6-P is now that of carbon 3 of S-7-P and carbons 4 and 5 are also labeled because of the 1,2 labeling of E-4-P. The Gl-3-P is again labeled in carbon 1. This reaction is the reverse of the transaldolase reaction as it is usually observed [Horecker & Smyrniotis (16)]. The enzyme occurs in spinach leaves [Gibbs & Horecker (31)] and is thought to be reversible.

Bassham *et al.* (13) point out that a reaction sequence similar to the one outlined here must lead to S-7-P which is equally labeled in carbons 3, 4 and 5. However, they report that after only 0.4 sec. photosynthesis in the presence of $C^{14}O_2$ the labeling of carbon 4 is significantly lower than of carbons 3 and 5. Since carbon 4 of S-7-P arises from carbon 3 of F-6-P, the authors conclude that in the initial labeling of F-6-P carbon 3 is less radioactive than carbon 4. Dilution of the label in carbon 3 by the exchange of carbons 1, 2 and 3 of F-6-P with endogenous DAP, that is, incomplete equilibration of the two triose phosphates, could account for the results of extremely short term experiments. Longer exposures to light and $C^{14}O_2$ permit more extensive equilibration and lead to virtually equal labeling of carbons 3, 4, and 5.

The further conversion of S-7-P is a second transketolase reaction (reaction g) with Gl-3-P. The products are R-5-P and Ru-5-P. The 3, 4, 5 labeling of S-7-P becomes a 1, 2, 3 labeling of R-5-P, and the labeled carbon 1 of Gl-3-P becomes carbon 3 of Ru-5-P. The formation of S-7-P from R-5-P and Ru-5-P, and the reversal of this reaction, have been described by Horecker *et al.* (30) for spinach leaves. The R-5-P can be converted into Ru-5-P by pentose phosphate isomerase (reaction h) which is known to be present in spinach leaves [Axelrod *et al.* (28), and Horecker *et al.* (30)]. There are therefore three reactions by which Ru-5-P is formed. The transketolase reactions (e and g) which give rise to two molecules of 3-labeled Ru-5-P, and the isomerase reaction (h) which forms one molecule of 1, 2, 3-labeled Ru-5-P. The observed radioactivity in Ru-5-P is therefore mainly in carbon 3 and the remainder equally distributed between carbons 1 and 2, in agreement with the observed results.

The Ru-5-P can be converted to Ru-1,5-P_2 by the ribulokinase reaction of Weissbach *et al.* (24), and the F-1,6-P_2 hydrolyzed to F-6-P and P_i by hexosediphosphatase. However, this reviewer wishes to speculate on the occurrence of a transphosphorylation from F-1,6-P_2 to Ru-5-P (reaction 11; in Figure 1, reaction j). Since two molecules of F-1,6-P_2 are available to

phosphorylate two out of three molecules of Ru-5-P, such a reaction would save two molecules of ATP.

$$\text{Ru-5-P} + \text{F-1,6-P}_2 \rightarrow \text{Ru-1,5-P}_2 + \text{F-6-P} \qquad 11.$$

Reaction 11 differs from a "mixed mutase" reaction like the one observed by Klenow (32) in that the phosphate group is transferred from carbon 1 of the fructose to carbon 1 of the ribulose, instead of from carbon 1 of the donor to carbon 5 of the acceptor. Another type of transfer of esterified phosphate groups which may be of significance here has been observed by Axelrod (33) and more recently by Morton (34). These authors observed a transphosphorylation catalyzed by phosphatases. Axelrod worked with an enzyme associated with acid phosphatase in plant tissues, while Morton observed transphosphorylations carried out by alkaline phosphatase of intestinal mucosa, milk, and various animal tissues. Morton found that kidney hexose diphosphatase does not catalyze transphosphorylations, but the hexosediphosphatase of green leaves has not yet been studied from this point of view. In the absence of any evidence for reaction 11 it can only be pointed out that this conservation of the phosphate ester bond energy represents a saving of two molecules of ATP per molecule Gl-3-P synthesized, and that such a saving may be required to carry out photosynthesis with the high efficiency that Warburg's (35) data indicate.

The Fuel of Photosynthesis

The synthesis of Gl-3-P by the reaction scheme outlined in Figure 1 can be expressed by the balance reaction

$$3\ CO_2 + 7\ ATP^{4-} + 6\ TPNH + 3\ H_2O \rightarrow \text{Gl-3-P}^{2-} + 6\ PO_4^{3-} + 7\ ADP^{3-} + 6\ TPN^{+} + 7\ H^{+} \qquad 12.$$

The net formation of Gl-3-P therefore requires the continuous generation of $TPNH^2$ and ATP.

Reduction of pyridine nucleotides.—It was suggested by Ochoa (36) that the reduction of the pyridine nucleotides, TPN in particular, is an essential part of photosynthesis. Subsequently, the reduction of DPN^2 and TPN by chloroplast fragments has been observed in several laboratories, and it has been possible to carry out a variety of net reactions by illuminating chloroplast fragments in the presence of the appropriate coenzyme, enzyme, and substrate [Vishniac & Ochoa (37); Tolmach (38); Arnon (39); Evans & Nason (40); and Hendley & Conn (41)]. It has been possible to observe TPN reduction in a soluble system derived from acetone dried chloroplasts which required added chlorophyll [Vishniac (42)], but nothing is as yet known concerning the mechanism of this reaction. The addition of chlorophyll to a colorless protein solution is reminiscent of Rodrigo's (43) experiments in which he added a chlorophyll solution to particles prepared from colorless leaves of a variegated *Pelargonium* and observed, to a limited ex-

tent, a Hill reaction with quinone. Calvin's suggestion that the conversion of light energy to chemical energy leads to the formation of reduced 6,8-thioctic acid [reviewed by Lumry, Spikes, & Eyring (2); Calvin (44)] might provide a pathway for TPN reduction via a TPN-linked thioctic dehydrogenase. A DPN-linked thioctic dehydrogenase has been described by Hager & Gunsalus (45). It is pertinent to mention here that the data on the concentration of pyridine nucleotides in green leaves obtained by Whatley (46) have recently been bettered by Anderson & Vennesland (47). An examination of leaves of bean, corn, tomato, parsley, pea, and spinach showed both DPN and TPN to be present in a concentration range of $1\text{-}2\times10^{-5}M$ (approximately 10 μg. per gm. fresh tissue). Duysens (48) has obtained data which indicate a rapid reduction of pyridine nucleotides in *Chlorella* and *Porphyridium* upon illumination.

Formation of ATP.—The intimate relationship between photosynthesis and phosphate esterification was perhaps first indicated by the experiments of Barrenscheen & Pany (49) who, twenty-five years ago, immersed *Elodea* plants in glucose solutions and observed a significant increase in hexosemonophosphates upon illumination. Ruben (50) specifically suggested that ATP, formed during the oxidation of reduced photosynthetic intermediates, participates in photosynthesis. The uptake of inorganic phosphate by photosynthetic organisms in the light, and its release in the dark, was followed by Gest & Kamen (51), Wassink *et al.* (52), and especially by Kandler (53). A light-dependent phosphorylation in *Elodea* has been found by Grube (54) in the absence of CO_2 fixation. Of particular significance are the findings of Strehler (55) that ATP formation is a very early event in photosynthesis and intimately related to it. Holzer (56) has shown that DNP[2], presumably by uncoupling phosphorylation, inhibits photosynthesis. In his experiments he used concentrations of DNP which did not affect respiration in the dark, and found that photosynthesis, as measured by O_2 evolution in the presence of CO_2, was inhibited. Improved experiments [Holzer (1)] with *Chlorella*, in which a pH of 5.4 favored penetration of DNP, showed an almost complete suppression of photosynthesis at a concentration of $5\times10^{-5}M$. DNP. Dark respiration was unaffected.

While the data cited provide evidence for a close connection between photosynthesis and ATP formation, there is nothing in them to indicate by what mechanism photosynthetic phosphorylation proceeds. Unless one wishes to postulate a unique photochemical mechanism [Emerson *et al.* (57)], the possibility of oxidative phosphorylation accompanying photosynthesis suggests itself. Zöllner (58) demonstrated the occurrence of oxidative phosphorylation in *Chlorella*. Vishniac & Ochoa (59) constructed a model system in which illuminated chloroplast fragments reduced DPN, the reoxidation of which by mitochondria of etiolated mung bean seedlings, led to ATP synthesis. It was suggested that in photosynthesis phosphorylation occurred in two phases. One, similar to the Hill reaction, in which a

photolytic split of water leads to oxygen evolution and the formation of reduced matter, most likely a coenzyme (reaction 13); and another (reaction 14) in which the reoxidation of the reduced compound is coupled to phosphorylation as in mitochondria [Lehninger (60)].

$$H_2O + DPN \rightarrow DPNH + H^+ + \tfrac{1}{2}\, O_2 \qquad 13.$$

$$DPNH + H^+ + \tfrac{1}{2}\, O_2 + 3\ ADP + 3\ P_i \rightarrow DPN^+ + H_2O + 3\ ATP \qquad 14.$$

In these experiments Vishniac & Ochoa (59) found that chloroplast fragments themselves were unable to phosphorylate, which did not preclude the possibility that intact chloroplasts might do so. Convincing evidence for phosphorylation in chloroplasts was obtained by Arnon *et al.* (61, 62) who found a net uptake of up to 16 μM of P_i[2] per hour by illuminated chloroplasts. The esterified phosphate could be recovered as ATP when AMP or ADP[2] were used as acceptors, or as G-6-P[2] when hexokinase and glucose were also present. This photosynthetic phosphorylation at first appeared dependent on oxygen, but it was found later that in the presence of several cofactors phosphorylation in the light proceeded under anaerobic conditions. Maximal rates of P_i esterification were attained in the presence of ascorbic acid, FMN, menadione (the active moiety of vitamin K), and Mg^{++}. The activity of these same chloroplast preparations in a Hill reaction with quinone, combined with an oxygen requirement when cofactors are absent, strongly suggest that photosynthetic phosphorylation is indeed an oxidative phosphorylation preceded by a photochemical reduction. While the biochemical sites of action of the added cofactors are not yet known, these stimulations are reminiscent of the experiments of Mehler (63) in which ascorbic acid oxidation by chloroplasts proceeded most rapidly when the chloroplasts had previously reduced quinone. Since menadione can act as a Hill reagent [Wessels & Havinga (63a)] it is tempting to speculate that menadione undergoes reduction in the light followed by oxidation coupled to phosphorylation. The reduction of menadione (reactions 15 and 16) may be mediated by a DPN-linked enzyme similar to the quinone reductase of Wosilait & Nason (64).

$$H_2O + DPN^+ \rightarrow DPNH + H^+ + [O] \qquad 15.$$

$$DPNH + \text{menadione} + H^+ \rightarrow DPN^+ + \text{menadiol} \qquad 16.$$

The product [O] can be gaseous oxygen re-used *in situ*, a precursor of oxygen, or oxidized cytochrome. The menadiol may then undergo an oxidation (reactions 17 and 18) catalyzed by a flavoprotein and stimulated by ascorbic acid, resembling the ascorbic acid stimulation observed by Kern & Racker (65) and Nason *et al.* (66).

$$\text{menadiol} + FMN \rightarrow \text{menadione} + FMN \cdot H_2 \qquad 17.$$

$$H_2 \cdot FMN + [O] \xrightarrow[\text{ascorbic acid}]{} FMN + H_2O \qquad 18.$$

Part or all of the reaction sequence 16, 17, 18 may be coupled to phosphory-

lation. On the basis of studies of dicumarol inhibition Martius & Nitz-Litzow (67) had previously postulated the participation of vitamin K in oxidative phosphorylation. Following these authors, a possible role of vitamin K in photosynthesis had been suggested by Wessels (68) who envisioned vitamin K as a natural hydrogen acceptor in the photolysis of water, as a hydrogen transfer agent to the pyridine nucleotides, and as an intermediate in oxidative phosphorylation. The presence of a phytol chain in both chlorophyll and vitamin K and the concentration of vitamin K in chloroplasts suggested to Wessels a periodic arrangement of chlorophyll and vitamin K molecules which could function as a "photochemical unit."

A further property of the intact chloroplasts in Arnon's experiments is their ability to fix CO_2 in the light [Arnon *et al.* (61)]. The uptake of CO_2 was measured by the incorporation of radioactivity by the chloroplasts when $C^{14}O_2$ was administered. Although the products of this CO_2 fixation have not been identified, it is likely that 3-PGA and related compounds are formed similar to the products of CO_2 fixation by particulate preparations in the experiments of Fager (12). It was found that CO_2 fixation was partly inhibited by the addition of P_i. If we assume ATP formation by phosphorylative oxidation of reduced pyridine nucleotides, and CO_2 fixation by the utilization of both ATP and TPNH, then this effect can be, in part, explained by the competition for TPNH. Thus, in the presence of phosphate acceptors, P_i would favor the reoxidation of TPNH resulting in phosphorylation, whereas a low P_i concentration might channel some TPNH into the reduction of 3-PGA. Arnon and his collaborators suggest that a certain biological organization is required for phosphorylation and CO_2 fixation. Leaf homogenates and intact chloroplasts will carry out a Hill reaction, photosynthetic phosphorylation, and CO_2 fixation, while chloroplast fragments in their hands showed a Hill reaction as the only photochemical activity.

In a recent paper Ohmura (69) described a preparation of chloroplast fragments which carries out oxidative phosphorylation. The preparation of Ohmura differs from conventional preparations only in the addition of citrate and ethylenediamine tetracetic acid. Particles prepared in the presence of these chelating agents took up P_i in the dark under air with citrate, malate, and other acids as substrates. Phosphorylation could be detected by ATP formation when ADP was added as an acceptor, or by the formation of G-6-P in the presence of glucose and hexokinase. It appears, therefore, that phosphorylation need not be restricted to intact chloroplasts, but that chloroplast fragments can carry out the same reaction. The finding of Ohmura that the chloroplast particles readily oxidized several members of the tricarboxylic acid cycle strongly suggests the existence of a complete tricarboxylic acid cycle in these particles. It is doubly important for this reason to establish the identity of the products of CO_2 fixation in Arnon's experiments with intact chloroplasts, since the additional possibility exists that radioactivity was incorporated in Krebs cycle compounds. Although it is known [Calvin (44)] that during photosynthesis the tricarboxylic acid

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cycle is largely inhibited, this inhibition should be experimentally verified for these particles also.

The occurrence of oxidative phosphorylation in photochemically active particles is supporting evidence for the view that the ATP required for photosynthesis is formed by oxidative phosphorylation. Oxygen or a similar hydrogen acceptor should therefore be required for photosynthesis. This hydrogen acceptor could be furnished by the oxidized product of the photolysis of water (reaction 15), which could be either free oxygen, a precursor of oxygen, or some other oxidized compound. Warburg (70) has reported that *Chlorella*, preincubated under strictly anaerobic conditions, will not photosynthesize until air is admitted. Hill & Whittingham (71), who studied the induction period of photosynthesis in *Chlorella* after aerobic and anaerobic incubation in the dark, concluded from their data that a partial pressure of at least 2 mm. Hg of oxygen is required for photosynthesis. On the other hand, Allen (72) interpreted data obtained with *Scenedesmus* to the effect that oxygen was not required to initiate photosynthesis. Under anaerobic conditions (10^{-6}mm. Hg of oxygen) a 0.5 msec. light flash led to oxygen evolution in a Hill reaction, but not in photosynthesis unless the algae had previously been exposed to a longer light flash (25 msec.). These data may well mean that some hydrogen acceptor, possibly a precursor of oxygen, can fulfill the oxygen requirement of photosynthesis. As has been pointed out above, such a hydrogen acceptor could function in oxidative phosphorylation, which would not be required for the Hill reaction. This internal hydrogen acceptor may be oxidized either by a reaction with gaseous oxygen or by a reaction with the oxidized product of the photolysis of water. From this point of view there is no discrepancy in principle between those workers who claim a requirement for oxygen and those who find that preillumination under anaerobic conditions is sufficient.

If ATP is required, not only for the initiation, but for the maintenance of photosynthesis, then oxidative phosphorylation should continue during photosynthesis. Data which may lend support to this view have been obtained by Brown (73). Using oxygen enriched with O^{18}, Brown has shown that the rate at which O^{18} is incorporated into water during respiration in the dark continues linearly during photosynthesis, so that it would appear that the rate of respiration in green algae is independent of photosynthesis. The observation of Calvin and his collaborators [reviewed in (44)] that the tricarboxylic acid cycle is inhibited during photosynthesis, leaves the possibility that the oxygen uptake is entirely devoted to the reoxidation of some of the pyridine nucleotides reduced in photosynthesis. Such oxidation would lead to ATP formation. With the blue-green alga *Anabaena*, Brown & Webster (74) found that the rate of respiration during photosynthesis varied with the partial pressure of oxygen. Very low concentrations of oxygen (0.5 per cent) led to partial inhibition of respiration, while higher concentrations of oxygen stimulated respiration in the light.

Particles prepared from the purple bacterium *Rhodospirillum rubrum*

were shown by Frenkel (75) to carry out a photosynthetic phosphorylation. The esterified P_i could be recovered as ATP when ADP was used as the acceptor in substrate amounts, or as mannose-6-phosphate when mannose, yeast hexokinase, and catalytic amounts of ADP were used. The phosphorylation by the washed particles was stimulated over twofold by the addition of α-keto glutarate. In the absence of other information the reviewer wonders whether perhaps this stimulation is similar to the stimulation obtained with citrate and ethylenediamine tetracetic acid by Ohmura (69). In this connection it is of interest to examine the work of Johnston & Brown (76) who confirmed, by the use of O^{18}, the observations of van Niel [reviewed in (77)] that *R. rubrum* does not evolve oxygen in photosynthesis and that respiratory oxygen consumption is inhibited in the light. It must be concluded from this paper that if *Rhodospirillum* generates ATP by oxidative phosphorylation that the hydrogen acceptor is derived from the oxidized product of the photolysis of water. The observation of Frenkel (75) that oxygen was not required in the photosynthetic phosphorylation by particles is in agreement with this view.

Photochemical Oxidations

Since Hill & Hartree (78) reviewed the hematin compounds in plants two years ago, a great deal of information has been published concerning the possible role of cytochromes in photosynthesis. The critical review of Smith (79) on bacterial cytochromes has greatly stimulated investigations on respiratory pigments among microorganism in general. Hill (80) has recently identified another cytochrome component in plants. Barley and *Chorella* contain a cytochrome of the *b* type, which Hill has named cytochrome-b_6, and which is present in larger amounts than cytochrome-*f*. At pH 7 cytochrome-b_6 has a E_0' of -0.06 V as compared with the $+0.365$ V of cytochrome-*f*.

Elsden *et al.* (81) have reported the occurrence of a new soluble cytochrome resembling cytochrome-*c*. This cytochrome occurs in large amounts in the photosynthetic bacterium *R. rubrum*. A later paper by Vernon & Kamen (82) gives the distribution of this or a closely related cytochrome among a variety of bacteria. It is present in all photosynthetic and nonphotosynthetic aerobic bacteria which were examined. It is absent in obligately anaerobic heterotrophs as for instance, *Clostridium pasteurianum*. The same authors [Kamen & Vernon (83)] find that this bacterial cytochrome cannot be oxidized by cytochrome-*c* oxidase obtained from animal tissues. However, the purple bacteria contain an oxidase specific for this cytochrome, a pyridine nucleotide linked reductase, and, in addition, a light-activated cytochrome oxidase. Since *Rhodospirillum* is a facultatively anaerobic organism capable of respiring in the dark with oxygen, the existence of a cytochrome is perhaps not surprising. The relationship of this cytochrome to photosynthesis is suggested by the existence of the light-activated oxidase

and also by the finding that an obligately anaerobic photosynthetic bacterium, *Chlorobium limicola*, possesses the same cytochrome together with the cytochrome reductase, the "dark" oxidase, and the "light" oxidase [Kamen & Vernon (84)]. These findings attach added significance to the earlier observation of these authors [Vernon & Kamen (85)] of a light dependent oxidation of reduced cytochrome-*c* in cell free preparations of purple bacteria. Since these organisms do not evolve oxygen in photosynthesis it is suggested that the cytochrome is oxidized in the light by the oxidized product of the photolysis of water and that the reduction of the cytochrome is a pyridine nucleotide-linked respiratory dehydrogenation of an external substrate which these organisms require for photosynthesis. The inhibition of oxygen consumption in the light may be explained by a competition of the "light" oxidase for the site occupied in the dark by the "dark" oxidase. Evidence has accumulated that the illumination of photosynthetic organisms rapidly leads to the oxidation of a cytochrome or cytochromes. A change in cytochrome absorption in *Chlorella* upon illumination has been observed by Lundegårdh (86). Chance & Smith (87) show the oxidation of a cytochrome component which occurs in *R. rubrum* upon illumination. Duysens (88), working with the same organism, finds a rapid cytochrome oxidation in the light, and reduction in the dark. Similar observations were made by Duysens (89) on *Chlorella* and *Vallisneria*. Duysens (48) also succeeded in correlating the oxidation of cytochromes with the reduction of pyridine nucleotides which occurs upon illumination of *Chlorella* and *Porphyridium*.

Vernon & Kamen (90) have carried out a comparative study of photo-oxidation in purple bacteria and green plants. Like Mehler (63), who observed the photo-oxidation of ascorbic acid by quinone-treated chloroplasts, these authors believe that a hydrogen-donating redox couple (e.g., quinone-ascorbate) competes with the oxygen-evolving system for the oxidized product of the photolysis of water, while the reduced product consumes oxygen. Since the purple bacteria do not evolve oxygen, such photo-oxidations may be a normal manifestation of the Hill reaction in preparations obtained from purple bacteria. Using spinach leaf homogenates and extracts of *R. rubrum* prepared by sonic oscillation, Vernon & Kamen studied the photo-oxidation of an ascorbic acid-2,6-dichlorophenolindophenol couple. The ascorbate-indophenol couple was readily oxidized by plant and bacterial preparations in the light, while an ethanol-catalase system was not. The simultaneous addition of both the redox couple and ethanol plus catalase to either plant or bacterial extracts doubled the amount of oxygen consumed, but not the oxidation rate, and led to the formation of one mole of acetaldehyde for every two moles of dehydroascorbic acid. The dependence of ethanol oxidation on ascorbate oxidation, as well as the fixed ratio of the products, suggested to the authors that both oxidations were closely linked, rather than merely occurring simultaneously. Hydrogen peroxide was not formed in

these reactions, as judged by the failure of added hydrogen peroxide to oxidize ascorbic acid. Under the conditions of this experiment both plant and bacterial preparations carried out a Hill reaction in which oxygen acted as oxidant, thus emphasizing the close similarity of plant and bacterial photosynthesis. Both plants and purple bacteria in the light appear to produce similar or identical oxidation products of water, but plants possess the added ability of evolving oxygen from this product.

Photochemical Pigments

Chlorophyll.—Smith has continued his studies on the conversion of protochlorophyll to chlorophyll. He has shown that in etiolated barley leaves (91) the ability to evolve oxygen in the light parallels the development of chlorophyll. He has also examined the rate of chlorophyll formation at various temperatures [Smith & Benitez (92)]. A notable contribution to the study of chlorophyll has been the development of a simple procedure for the crystallization of chlorophyll-*a* and bacteriochlorophyll (Jacobs *et al.* [93)]. Holt & Jacobs (94, 95) have carried out extensive comparative studies on the spectral properties of various chlorophylls and their derivatives. Ethyl chlorophyllides *a* and *b*, their corresponding pheophorbides, bacteriochlorophyll, and methyl bacteriochlorophyllide have been examined in a variety of solvents under different conditions. Evstigneev & Gavrilova (96) have presented additional spectroscopic data to support their view that chlorophyll is the primary reduced product of photophynthesis. An ingenious application of the Molisch reaction, the reduction of silver nitrate to black metallic silver by illuminated plant particles, suggested to Thomas *et al.* (97) that chlorophyll is restricted to the lamellae of the chloroplasts. Since the action spectrum of the Molisch reaction coincided with the chlorophyll absorption spectrum, and since electron micrographs showed silver deposition in the lamellae, the authors concluded that this established the distribution of chlorophyll in chloroplasts.

Carotenoids.—Engelmann's contention in 1882 that carotenoids absorb light which can be used in photosynthesis has, in principle, been borne out by many studies [Blinks (98)]. A new aspect of this problem has recently been reported by Warburg *et al.* (99). It was found that the addition of a blue-green light source to the incandescent light usually used in experiments on *Chlorella* greatly increased the quantum efficiency of the algae during photosynthesis. When *Chlorella*, grown under incandescent light, was used in experiments designed to measure photosynthetic efficiency, a quantum yield of approximately 10 was obtained when incandescent light was the only light source. The addition of a cadmium lamp (major emission at 468, 480, and 509 mμ) rapidly raised the quantum yield to about 4, even though the energy emitted by the cadmium lamp was only a fraction of a per cent of the total illumination. The alternate addition and removal of the cadmium lamp resulted in a rapid and reversible fluctuation of the quantum yield

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between 4 and 10. The response of the algae occurs within minutes, with the carbon dioxide uptake varying more rapidly than the oxygen evolution. A more general account [Warburg & Krippahl (100)], in addition to some vituperation aimed at Franck and Rabinowitch, contains more information on this remarkable effect. Although a complete action spectrum of the increase in quantum yield has not yet been obtained, Warburg finds little effect at 436 mμ, most in the 468 to 509 mμ region, and none at 546 or 644 mμ. Since a light-catalyzed reversible reaction appears to be involved, he concludes that it is mediated by a carotenoid pigment, in analogy with reactions of the visual pigments. Low concentrations of cyanide, which do not inhibit photosynthesis, block the light-catalyzed increase in photosynthetic efficiency, but *o*-phenanthroline does not. These findings add to the number of cofactors required for photosynthesis. According to Warburg, the list now includes: (*a*) pyridine nucleotide, required for the reduction of carbon dioxide; (*b*) a carotenoid pigment, required for the transfer of hydrogen from water to the pyridine nucleotide; (*c*) a heavy metal, required for "carotene mutase," the enzyme catalyzing the conversion of the inactive carotenoid into the active form; (*d*) a heavy metal, required for the oxidative "back reaction" which supplies two-thirds of the photosynthetic energy; and (*e*) iron, required for oxygen evolution. Zinc and chloride ions are not mentioned in this paper.

Methods

Algal chloroplasts.—Attempts at isolating chloroplasts from algae have in the past been generally unsuccessful. Punnet & Fabiyi (101) have obtained active chloroplast fragments, as judged by a Hill reaction, from a variety of microscopic algae, including *Chlorella*. The algae were mixed with alumina and the paste forced under high pressure through a small opening. Photochemically active chloroplast fragments from *Chlorella* were obtained independently by Hill *et al.* (102) who ruptured the algal cells in a Mickle disintegrator. The fragile chloroplasts of red algae were for the first time isolated in a photochemically active state by McClendon (103). Sucrose, which has been used since Engelmann's time to maintain osmotic pressure in the isolation of chloroplasts from green plants, inactivates the chloroplasts of red algae, apparently by penetrating the plastid membrane and disorganizing the interior. The use of a commercial polyethylene glycol of molecular weight 2500 (Carbowax 4000) instead of sucrose, leads to the isolation of algal chloroplasts active in the Hill reaction. Chloroplasts of green plants which generally lose photochemical activity on standing in dilute buffers, were found frequently to remain active for considerable periods when suspended in Carbowax 4000.

Other methods.—Rosenberg (104) has developed a method for rapid and sensitive measurements of photosynthesis, based on the change in pH which occurs when photosynthesis takes place in a bicarbonate solution. The reaction vessels and circuits required for the continuous recording of redox

potentials in photosynthesis or Hill reaction have been described by Spikes *et al.* (105). The forthcoming treatise *Methods in Enzymology* will contain a chapter by Gorham on the preparation of chloroplasts and chloroplast fragments, and one by Vishniac on methods of studying the Hill reaction [Colowick & Kaplan (106)].

Additional Observations

Tolbert & Zill (107) have succeeded in extruding from the cells of *Chara* and *Nitella* whole protoplasts which were able to photosynthesize. Such preparations permit the addition of various substrates to a photosynthesizing system without interference from a cell wall of low permeability. The extruded protoplasts differed from the intact cells in that sucrose and the intermediate sugar phosphates were formed in relatively smaller amounts, while proportionately greater amounts of malic acid and aspartic acid appeared. The authors conclude that the extrusion of the protoplasts interfered with the "C_2 acceptor cycle" which normally leads to the formation of 3-PGA. Such interference may be interpreted as an impairment of the phosphorylating mechanism which is notoriously sensitive. The lack of ATP would interfere with the resynthesis of Ru-1,5-P_2, while permitting the accumulation of additional amounts of malic acid.

Siegel (108) has shown that the photosynthetic oxidation of acetone by the purple bacterium *Rhodopseudomonas gelatinosa* proceeds via an intermediate CO_2 fixation. The reaction sequence is

$$\text{Acetone} + CO_2 \rightarrow \text{Acetoacetate} \rightarrow 2\ \text{Acetate}$$

and proceeds with an overall change in free energy of -9 kcal. Verduin (109) has calculated from data in the literature the compensation point in photosynthesis with respect to carbon dioxide. When light is not limiting respiration counterbalances photosynthesis at a partial pressure of carbon dioxide close to 7×10^{-5} atm.

Horwitz (110) found that D_2O inhibited photosynthesis and the Hill reaction with quinone in *Chlorella*, but that these inhibitions differ qualitatively. No definite conclusions were drawn by the author, who suggests that the combination of quinone and D_2O may have a different effect on the Hill reaction than D_2O alone has on photosynthesis. Frenkel & Lewin (111) have found that *Chlamydomonas moewusii* adapts within a few minutes to the use of hydrogen in photoreduction, the light-dependent simultaneous uptake of carbon dioxide and hydrogen. Other algae usually require several hours for this adaptation. *Chlamydomonas* appears therefore to be the alga of choice for the study of the relationship of hydrogen metabolism to photosynthesis. Gilmour *et al.* (112) have obtained kinetic evidence for a so far unidentified intermediate in the Hill reaction. Boichenko & Baranov (113) suggest that an iron-containing organic compound participates in photosynthetic carbon dioxide fixation.

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General Comments

For the past several years the study of photosynthesis has increasingly occupied public attention. Students of photosynthesis have been asked to supply information to the public press or to comment on such information. While a certain distortion in public reporting cannot always be avoided, it is regrettable that popular writers have interpreted in an extravagant manner statements about synthesis of food without green plants, in the future [Plumb (114)]. In the days of extensive public support it is important to supply public information. It is equally important to do so without raising false hopes with sensational, misleading, and exaggerated statements.

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EFFECT OF ECOLOGICAL FACTORS ON PHOTOSYNTHESIS[1,2]

By Moyer D. Thomas
Stanford Research Institute, Stanford, California

In the complex system involving the relationships of a plant to its environment, many interdependent factors come into play and mutually influence each other as they affect the various functions of the plant, including the over-all photosynthetic process. The principal ecological factors to be evaluated in photosynthesis are intensity, quality, and duration of the light; carbon dioxide or carbonate concentration; temperature; soil moisture; relative humidity; age of the leaves; general nutritional level; concentration of particular nutrients like nitrogen, phosphorus, or potassium; pH of the nutrient supply; and toxic compounds in the air or soil. Light and carbon dioxide, being directly involved in photosynthesis, cause immediate responses when they change. The temperature effects vary with conditions, often being rather slight, but at times very important. Soil moisture and relative humidity affect the turgor of the leaves, which, in turn, influences the stomatal apertures which regulate gas absorption by the leaves. All of these factors and the others listed may operate indirectly by changing the morphology of the leaf, or by modifying the enzyme systems and the vigor of the plant.

The literature on the photosynthesis of the higher plants under natural conditions is limited, owing to the inconvenience and difficulty of making adequate measurements of the carbon dioxide intake or oxygen evolution of the leaf. Such measurement can best be carried out by passing a measured volume of air or other gas mixture over an illuminated leaf, plant, or plot, and determining the oxygen or carbon dioxide concentration of both the ingoing and outgoing gas. Measurement of oxygen exchange in air is hardly practicable because of the large percentage present and the small differences which would have to be accurately determined. On the other hand, the carbon dioxide level is low, and simple, accurate methods are available for its analysis. Some studies have been made with air enriched or depleted in oxygen and carbon dioxide. Yield studies provide an indirect and approximate measure of photosynthesis, as does also the increase in weight due to illumination of detached leaves or leaf disks, or even carbohydrate analysis of the leaves. Gregory and co-workers (17, 18, 22) measured the "net assimilation rate." This is the increase in weight of the plant per unit leaf area per unit time. It is found by determining at intervals the weight and leaf area of sam-

[1] The survey of the literature pertaining to this review was concluded in January, 1954.

[2] The following abbreviations will be used: μ^2, average pore area; ft-c, foot candle; p.m.d., pore mean diameter; v.p.d., vapor pressure deficit.

ples of the vegetation on a plot. They consider it an excellent practical measure of photosynthesis, that is particularly useful in ecological studies.

Heinicke and his co-workers (24, 25) measured the photosynthesis of large apple trees throughout the whole growing season and their analytical methods have been used by several other investigators (7, 10, 64). Thomas & Hill (59, 60, 61) employed recording carbon dioxide analyzers to measure gas damage by common air pollutants and made many observations of the photosynthesis of plants under field conditions. Katz, Ledingham, & Harris (29) also used recording carbon dioxide analyzers in sulfur dioxide fumigation studies. Infrared spectroscopy (27, 43) is an excellent method of measuring carbon dioxide exchange if the equipment is made sufficiently sensitive.

The photosynthesis of water plants, particularly the algae, mosses, and other small submerged forms, has been extensively measured in the Warburg apparatus. From this work, often undertaken for other purposes, an extensive literature has developed on the ecological relationships of aquatic plants, some of which has a bearing on the ecology of land plants.

Light as an ecological factor and its measurement was reviewed in 1935 by Shirley (48). Most of the other recent reviews on photosynthesis are concerned with the chemical aspects of the process. Rabinowitch (41) discussed ecological factors in Volume II of his book.

Light and Carbon Dioxide

Saturation values.—The general pattern of the response of photosynthesis to changes in light intensity and carbon dioxide concentration is depicted in the old Blackman chart of limiting factors, but with a more or less rounded curve when the limit is approached rather than a sharp angle when the limit is reached. This is strikingly shown in the experiments of Hoover, Johnston, & Brackett (27), who grew a wheat plant under controlled conditions in a closed system, circulating the air from the plant chamber through a long cell with rock salt windows where the carbon dioxide concentration was measured continuously and automatically by absoprtion of infrared light. When photosynthesis is plotted as ordinate against carbon dioxide concentration as abscissa, photosynthesis increases at first in a steep, straight line with constant light intensity and increasing carbon dioxide concentration; then it increases less rapidly as carbon dioxide saturation is approached, finally assuming a horizontal direction. At successively higher light intensities, the steep straight portion of the curve is lengthened without change of slope and the horizontal portion is successively elevated. In one experiment, the curves indicate that saturation occurred at 1200 p.p.m. CO_2 with 947 ft-c. From the slope of the steep straight line, it appears that the rate of photosynthesis is doubled by doubling the carbon dioxide concentration but, from the over-all slope of the curve, the latter would need to be multiplied by about 2.5 times to double the photosynthesis. In another experiment, saturation was practically complete at 820 p.p.m. CO_2 and 1000 ft-c. Maximum assimilation in both experiments was linearly proportional to the light intensity up

to 1000 ft-c. Increasing the light to 2000 ft-c increased the assimilation only 25 per cent and the curve did not form a level plateau with high carbon dioxide concentrations but trended slowly upward after saturation was apparently reached. Evidently, the photosynthetic apparatus of the plant has a definite capacity limitation for reducing carbon dioxide.

Steemann-Nielsen (53) has emphasized that this pattern is generally applicable. Even water plants, which can reduce only carbon dioxide molecules but not bicarbonate ions, as well as those which can reduce bicarbonate ions, conform to the pattern. He plotted photosynthesis as the percentage of the maximum obtainable under optimum conditions as ordinate against free carbon dioxide concentration as abscissa (no bicarbonate was present in these experiments). The curves obtained were similar, but not coincident, to those of Hoover, Johnston & Brackett, for different types of plants. Maximum photosynthesis occurred with 500 p.p.m. CO_2 in *Hormidium*; with 1500 p.p.m. in wheat [data of Hoover *et al.* (27)]; and with 11,000 p.p.m. in *Myriophyllum spicatum*. These concentration differences were explained by differences in the rate of diffusion of the free or dissolved gas into the leaves or cells. For example, in the small-celled alga *Hormidium* a diffusion distance of only 1 μ is required for carbon dioxide to reach the chloroplasts, whereas in the higher aquatic plant *Myriophyllum* the diffusion distance may be 40 μ. In land plants diffusion takes place in the gas phase, which is much more rapid than diffusion in the liquid phase. Steemann-Nielsen (56) also found that *Chorella pyrenoidosa* attained maximum photosynthetic rate with 500 p.p.m. carbon dioxide but that more than about 10,000 p.p.m. reduced photosynthesis by blocking the respiratory mechanism in the light, but not in the dark.

Bonde (5a) observed that assimilation of carbon dioxide by excised leaves of *Sinapis alba* under optimal illumination and constant temperature was a logarithmic function of the carbon dioxide concentration. Most of the experimental curves of assimilation versus carbon dioxide concentration discussed by Rabinowitch (41) and Steemann-Nielsen (53) are really exponential. A notable exception is the curve of Emerson & Green (13) for *Chlorella*, which conforms exactly to the ideal Blackman pattern. The curves of Thomas & Hill (61) which employed higher concentrations of carbon dioxide than usual (up to 3700 p.p.m. in full sunlight) were still not carried to concentrations sufficiently high to reach saturation, and their overall shape was not indicated. It is of interest to note that the ability of a leaf to utilize carbon dioxide depends upon its previous history. Healthy plants growing under field conditions probably have greater capacity for assimilation than greenhouse plants, chlorotic plants, or excised leaves.

The curves obtained when photosynthesis is plotted against light intensity at constant carbon dioxide concentration are quite similar in shape to the photosynthesis-carbon dioxide curves at constant light intensity. A number of such curves have been published in recent years for crops, trees, and other higher plants growing under field conditions. Light saturation as meas-

ured in the field depends on the orientation of the vegetation and the time of year. For example, a leaf exposed perpendicularly to direct sunlight should require less intense light for saturation than a tree or plot in which some of the leaves are turned at various angles to the sun or are shaded by other leaves. In plots also, late-season shading may be greater than the shading in midsummer, but the phototropic orientation of the leaves may modify the results. From the data of Heinicke & Childers (24), light saturation values measured on a horizontal surface for a young apple tree of bearing age were about 4400 ft-c in June, July, and August, and about 4050 ft-c in September and October. Light saturation of directly illuminated corn leaves was about 2500 ft-c according to Verduin & Loomis (64). Thomas & Hill (61) found light saturation of alfalfa plots at 4700 ft-c during the summer and 3400 ft-c in the autumn. These values correspond to 5050 ft-c and 4900 ft-c at normal incidence. Light saturation for vegetative wheat in June and July was 5300 ft-c and for sugar beet in August to October, 4400 ft-c. Chapman & Loomis (7) measured paired potato leaves at right angles to the light and observed light saturation at 3000 ft-c with normal air, at 4200 ft-c with air enriched to 0.06 per cent CO_2, and at 5200 ft-c with 0.15 per cent CO_2. Photosynthetic rates under these conditions were respectively, 1.4, 3.2, and 5.0 gm. CO_2/m^2 leaf area/hr., equivalent to 0.96, 2.2 and 3.4 gm. glucose/m^2/hr.

Effect of shading.—Eaton & Ergle (11) noted that among irrigated cotton plants 110 to 160 cm. tall in California, the light intensity at half height was reduced to about 30 per cent, and at ground level to less than 5 per cent of the intensity above the plants. When the plants were shaded with muslin so as to reduce the light intensity to 32 per cent of full exposure starting at the beginning of flowering in late June, the concentration of sugar plus starch was reduced in the leaves by 24 per cent, in the main stems by 38 per cent, and in the 14-day-old bolls by 8 per cent, but the yield of seed cotton was reduced by 47 per cent owing to heavy shedding of the bolls. The growth reductions during the shading period from late June to August 21 were estimated to be 40 per cent for the leaves plus stems and 50 per cent for the bolls. Assuming an average incident intensity of about 6000 ft-c, 68 per cent absorption of light by shading, and 40 to 50 per cent reduction of maximum photosynthesis, light saturation for this cotton should be about 3000 to 3500 ft-c.

Blackman & Wilson (2) studied the effect of shading on the net assimilation rates of ten species of plants. They determined the total dry matter and leaf area at intervals of a few days by repeated sampling from the plots. The increments were net assimilation rates expressed as grams of dry matter/100 $cm.^2$ leaf surface/week. Portions of the plots were shaded to reduce the light intensity by one-third to nine-tenths of daylight. Plotting the leaf area per plant against the logarithm of the light intensity yielded a family of straight lines of different slopes converging at a point, for full daylight and for each of the corresponding reduced light intensities produced by the shade. Straight lines were also obtained when the net assimilation rates were plotted against

the logarithm of the light intensity. The intercept on the zero ordinate of the latter lines gave the compensation point where respiration balances photosynthesis.

It was concluded that "reductions in net assimilation" caused by shading were similar in all ten species studied. Irrespective of whether the species were normally associated with shady habitat (*Geum urbanum*) or grew in the open (*Helianthus annuus* or *Trifolium subterraneum*), the net assimilation during the season of active growth was related linearly to the logarithm of the light intensity. These relations held regardless of the average light intensity during a given period when the measurements were made—when, for example, the assimilation of sunflowers in full daylight was a maximum of 0.98 gm./100 cm.^{2}/week or a minimum of 0.30 gm. There was little change in activity during most of the growing season but in October and November, when growth was very slow, the logarithmic relationship failed and maximum assimilation occurred at one of the lower light intensities. Age of the plant had no effect from May to September. The constants of the net assimilation-logarithm of light intensity equations varied with the species. With sunflower and buckwheat the effects of shading were small. With barley the effects were larger. In the low temperatures of September vetch assimilated more rapidly than buckwheat. Under conditions of waterlogging due to too much rain and poor drainage, the assimilation rates of peas were consistently less at all intensities than those of buckwheat. Warmer conditions in the greenhouse increased the assimilation rate of tomato and sunflower but not of buckwheat. However, the seasonal variations of net assimilation of sunflower showed little correlation with temperature. The correlations with length of day and hours of sunshine were significant. The question of day and night temperatures was not considered. Possibly a detailed study of the latter would have been of interest.

The compensation point of eight of the ten species fell between 6 and 9 per cent of daylight intensity in normal air. Vetch had a value of 14 per cent and barley 18 per cent. Verduin (63) in summarizing the literature on the compensation point, indicated values ranging from 0.003 to 0.010 per cent carbon dioxide with full sunlight. Kok (30) has shown that with *Chlorella*, the slope of the oxygen evolution-light intensity curve above the compensation point is only about half as steep as that of the oxygen absorption-light intensity curve below the compensation point. Different types of reactions are evidently involved above and below the compensation point.

In another study, Blackman & Wilson (3) grew sunflowers with and without partial shade representing 1.0, 0.5, and 0.24 times daylight. After an initial period under these conditions the plants were transferred to other intensities, so that all combinations of initial high, medium, and low intensity with subsequent low, medium, and high light were obtained. This was an attempt to simulate woodland conditions in which deciduous and evergreen trees give various changes in shading when the leaves develop in the spring. It was found that, regardless of early lighting, the net assimilation in the

second period was the same linear function of the logarithm of the light intensity during the second period, even though some of the plant characters like leaf area were modified by the early lighting. Evidently, previous exposure did not modify appreciably the subsequent response of the plants to light.

Net assimilation.—Heath & Gregory (22) pointed out in 1938, on the basis of studies extending over 25 years, that the *average* net assimilation rates of a number of different plants grown in different parts of the world and having a wide range of leaf structures, were essentially the same during the period of rapid vegetative development. The short-time variations were due, in most cases, to changes in external conditions such as light intensity. Growth of barley at Rothamsted ranged from 0.418 to 0.658 gm. dry matter/100 cm.^{2}/week. A range of 0.413 to 0.720 gm. was observed for mangels and sugar beets at Rothamsted, cotton and tomato in South Africa, and tomato and cucumber under glass in England and Germany. Even a fourfold increase in the nitrogen supply of barley plants did not affect the net assimilation although it did change the size and succulence of the leaves. It was suggested that the internal processes in the leaves of acclimatized plants do not vary appreciably and that external conditions, such as light, temperature, length of day, stomatal density, etc., may compensate each other so that the net amount of carbon dioxide assimilated during vegetative growth per unit area of leaf surface after allowance is made for respiration, is everywhere about the same from the tropics to the arctic.

It could hardly be claimed that sufficient data were considered in Heath & Gregory's paper to establish the general validity of this hypothesis. Certainly, the net assimilation rates of individual plants vary over a wide range due to both internal and external factors. However, the work of Went (67, 68) on plants growing under optimal conditions, and of Hiesey *et al.* (26, 28) showing the adaptation of grasses to their environment, would suggest that an averaging and compensating effect on growth in different environments could occur.

Verduin (62) has compiled a table of photosynthetic rates under optimal near-natural conditions for the *Handbook of Biological Data.* The rates are calculated as micromols of carbon dioxide absorbed or oxygen evolved per hour on a number of weight, area, and volume bases. Expressed on the area of the leaves per square centimeter, the rates range from 2 to 4 for tropical, subtropical, temperate, and arctic sun plants. Alpine plants are assigned a value of 7, temperate shade plants a value of 1, and evergreen desert plants during the dry season a value of 0.1. The average rate of assimilation found by Heath & Gregory (22) of 0.4 to 0.7 gm. dry matter/100 cm.^{2}/week is equivalent to about 2.5 to 4 μM. CO_2/cm.^{2}/hr., assuming a 10-hour day and 44 per cent carbon in the dry matter. The rates should be increased 10 to 20 per cent to allow for losses due to respiration. The table appears to support the hypothesis of Heath & Gregory that assimilation rates are approximately the same everywhere for active plants in daylight.

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Calculating photosynthesis as micromols of carbon dioxide or oxygen per hour per milligram dry weight of the plant, temperate zone plants have a value of 1 while the algae range from 2 to 18, indicating that the algae are more efficient than land plants on a weight (or volume) basis. It is evident that the leaves of the higher plants have a substantial amount of tissue which is not involved in photosynthesis. A comment may be in order concerning the value of 18 micromols per hour per square centimeter of soil or water surface as compared with 7 for *Chlorella* in large-scale production. The former is calculated from alfalfa plots of Thomas & Hill (61). These plots received direct sunlight not only on the top but also on one or two sides so that the area of high illumination might have been as much as double the actual area of plot. This could reduce the apparent rate of photosynthesis from 18 to 9, due to better illumination of the lower leaves.

The high rate of photosynthesis indicated for alpine plants occurred in spite of a low total pressure of carbon dioxide due to altitude. One might expect that this would reduce the rate. Decker (9) showed that the rate of photosynthesis of tree seedlings was materially reduced by relatively small decreases in the carbon dioxide content of the air.

Very high light intensity.—Steemann-Nielsen (55), confirming earlier observations by Myers & Burr (36), found a decrease in the rate of photosynthesis with continued exposure to very high light, together with a temporary inhibition on changing from high to low light. For example, photosynthesis of the filiform green alga *Cladophora insignis* was constant at 280 ft-c for an indefinite period. After a 10-min. exposure to 9300 ft-c (100,000 lux), the subsequent rate at 280 ft-c was reduced about 50 per cent but the original rate was restored in about one hour. An hour's exposure to 9300 ft-c was accompanied by a gradual decrease in rate of 40 per cent. On lowering the light to 280 ft-c, photosynthesis fell below the compensation point but it increased to 30 per cent of the usual 280 ft-c value in two hours.

If, after two hours of exposure to 9300 ft-c, the plants were allowed to stand in the dark for 20 hr., they gradually recovered their full activity after about an hour at 280 ft-c. Similar experiments involving inactivation at 9300 ft-c, followed by reduction of the light intensity to either 1580 ft-c or 3250 ft-c, gave similar responses except that recovery of the full photosynthetic activity was more rapid at the latter intensities than at 280 ft-c. For example, after 50 min. at 9300 ft-c, 80 per cent of the activity was recovered in 40 min. at 3250 ft-c.

The author suggests that the gradual inhibition at 9300 ft-c is probably due to chlorophyll-sensitized photo-oxidation of one or more of the enzymes participating in photosynthesis, rather than to a partial temporary inactivation of the chlorophyll itself. Chemical rather than photochemical reactions limit the photosynthesis under these conditions. On lowering the light intensity, photochemical reactions again assume control, so that the lowered photosynthesis is likely to be due to inactivation of chlorophyll or to some other substance directly involved in the photochemical processes.

Continuous light.—Böhning (5) has studied apple leaves exposed to continuous illumination for periods as long as 17 days. Small trees were potted in April and allowed to grow outdoors until July. Then they were transferred to a thermostated box illuminated by Mazda lamps through a 2.25-in. water filter. At 6000 to 5600 ft-c from June 26 until July 13 there was no change in photosynthesis as measured by carbon dioxide exchange during the first six days. Photosynthesis was down 38.6 per cent during the last four days, which was only partly explained by a decrease of 5 per cent in the carbon dioxide concentration in the air. Marked bleaching had occurred. Evidently this treatment was too drastic. At 3200 ft-c from August 3 to 20 the rate of photosynthesis moved up to 130 per cent but there was an accompanying increase in carbon dioxide concentration of 14 per cent. At least this treatment did not harm the plants. Using uniform air from a tank, no change resulting from exposure was found from September 3 to 10 at 5200 ft-c. The following May, in plants with "shade" leaves grown under low light intensity in the greenhouse, photosynthesis at 3800 ft-c fell to zero in 19 days and severe bleaching developed. Evidently leaves developed in the shade are much more sensitive to continuous light than leaves developed in sunlight.

Plants have limitations not only in their exposure to light but also to carbon dioxide. Thomas & Hill (61) subjected tomato plants to 2000 to 3000 p.p.m. CO_2 and observed that extensive injury to the leaves developed in 1 to 2 weeks. The leaf injury was characterized by large necrotic areas and there was also inhibition of photosynthesis.

Light quality.—Scarth & Shaw (44) used *Petargonium* leaves and adjusted the light intensity to give just enough photosynthesis to compensate for respiration. Three colors were used: white light from a "daylight" lamp, green light filtered through a solution of chlorophyll in alcohol, and red light made by filtering white light through a glass filter which had a peak transmission at 6500 Å but a spread from 5490 to 8400 Å. Relative intensities required to cause equal photosynthesis were red 88, white 100, green 173. Stomatal apertures were practically identical with the three lights. The results agree with Paetz (40) who found red > blue > green, but not with Sierp (49) and Harms (19) whose order was green = blue > red. It is suggested that the latter used filters which transmitted too much inactive far-red and near infrared. The value for the red quoted by Scarth and Shaw is also somewhat too high. Paetz excluded the infrared.

Wassink & Stolwijk (57, 65) studied the effects of light of narrow spectral regions on cosmos, tomato, and *Iberis coronaria*. They used colored fluorescent lamps and glass filters for the blue, green, and red, and sodium lamps with an orange filter for the yellow. The blue light ranged from 3800 to 5000 Å with the peak at 4200 Å; the green from 4800 to 6000 Å with the peak at 5300 Å; and the red from 6100 to 7000 Å with the peak at 6600 Å. The yellow contained only a few per cent of energy beside the sodium lines. Total light intensity in all cases was 1350 ft-c. Dry weights of cosmos plants after exposure of 16 hr. per day for 33 days at day temperature of 20°C. and night temperature of 15°C. were as follows: blue, 259 mg.; green, 745 mg.; yellow,

518 mg.; red, 1144 mg.; and white, 710 mg. Stem elongation in 30 to 38 days in the three species was similar. In blue light, elongation was 70 to 100 per cent of that in white light; green, 120 to 170 per cent; yellow, 170 to 200 per cent; and red, 160 to 195 per cent. The data suggest that red light is most efficient in photosynthesis, followed by green. Blue light is least efficient. Yellow light causes as much elongation as red but only half the dry matter is incorporated into the plants.

In other experiments the authors supplemented 10 to 12 hr. of strong white light with 4 to 6 hr. of colored light of 24 ft-c intensity after the white lights were turned off. Violet, green, yellow, and red light had little effect on the plants, but blue (4000 to 5000 Å) and near infrared caused marked elongation of the stems of many different plants. The supplementary lighting was too weak to cause appreciable photosynthesis. Presumably, it promoted a low-energy light reaction affecting the growth process independently of the photosynthetic light reactions.

Stomatal Apertures

Because the initial step in photosynthesis involves the entrance of carbon dioxide into the leaf, a close relationship between the degree of stomatal opening and photosynthesis is often inferred. Scarth & Shaw (43, 44) have studied this relationship in *Pelargonium zonale*, using an infrared absorption method to measure photosynthesis, the epidermal stripping method to measure the pore mean diameter (p.m.d.), and a porometer method (calibrated by microscopic measurement of stomatal numbers and apertures) to measure the average pore area (μ^2). It was found that the stomata of *P. zonale* were generally closed in the dark. On illuminating the leaf, photosynthesis and both p.m.d. and μ^2 increased more or less together toward maximum values. In one experiment with 500 ft-c the photosynthesis and p.m.d. curves followed each other closely even when there was a temporary leveling off of both functions for no apparent reason. In other experiments with stepwise increasing or decreasing light intensity, the μ^2 curve was either above or below the photosynthesis curve but was similar in shape. In the changing illumination of natural daylight, photosynthesis and μ^2 curves were together while the p.m.d. curve was appreciably higher. If the stomata were closed when the leaves were illuminated, there was no photosynthesis until the stomata began to open. Thereafter, rate of photosynthesis kept pace with increasing apertures, suggesting that in the early stages of opening, at least, the rate of photosynthesis is controlled by the rate of diffusion of carbon dioxide through the partly opened stomata. This correlation between rate of photosynthesis and stomatal apertures is not supported by Mitchell (35) who found that at low relative humidity the rate of photosynthesis was about the same as at high humidity, though it was often noted that the stomata were closed in the former case but not in the latter. These observations were confirmed by a careful study of tomato and *P. hortorum*. Carbon dioxide absorption and starch formation were approximately the same at high and low relative humidities with the stomata open and closed. Increases

in acid-hydrolyzable carbohydrates were 59 and 41 per cent, respectively. No wilting or decrease in moisture content of the leaves occurred in these experiments. The reason for the apparent disagreement between Mitchell's results and those of Scarth & Shaw is not clear unless the stomata do not actually control carbon dioxide intake by the leaves and the gas can leak in around the closed guard cells in adequate amounts in some plants.

Effect of carbon dioxide.—Scarth & Shaw (44), repeating earlier experiments by Heath (20, 21, 23), also treated the plants with air free of carbon dioxide and found that the stomata tended to open wider than in normal air. Even a 12.5 per cent reduction of the carbon dioxide concentration caused a definite opening of the stomata at 50 ft-c and a much greater opening at 400 ft-c. Stepwise reduction of the carbon dioxide concentration to zero with low light intensity gave progressively greater open stomatal area. With high intensities, maximum opening was attained with partial removal of the carbon dioxide.

The foregoing results were interpreted to mean that stomatal movement is controlled primarily by the concentration of carbon dioxide inside the leaf. Opening is due to a reduction of internal carbon dioxide concentration either by photosynthesis, which would be most effective at high light intensities, or by reducing the external carbon dioxide supply at low light intensities. In the former case, there is a diffusion gradient inward, in the latter case outward. Other experiments conform to this pattern. A sublethal dosage of chloroform (0.005 cc./l.) at 1000 ft-c in normal air reduced photosynthesis to zero and also caused the stomata to close. After discontinuing the chloroform treatment, the stomata opened and photosynthesis returned to normal in 2 to 3 hr. In carbon dioxide-free air no stomatal closure occurred at 100 ft-c when the plants were treated with 0.008 cc. chloroform per liter. Evidently, the inhibition of photosynthesis by the chloroform permitted the carbon dioxide concentration in the leaf to rise, causing the stomata to close. This did not occur in carbon dioxide-free air because the internal carbon dioxide concentration was already depleted.

Alvim (1) suggested that the movement of the stomata is due to the reversible interconversion of starch and soluble carbohydrates in the guard cells, due to phosphorylase, as a result of changes in light and pH. No support could be found for the theory of colloidal swelling in the guard cells.

Water balance.—Opening of the stomata is also affected by the water balance of the leaves. Scarth & Shaw (44) cut the petioles of leaves that were adjusted to different light and humidity conditions: high or low light with large or small vapor pressure deficits (v.p.d.). Photosynthesis immediately began to fall, reaching zero in 30 to 90 min., depending on the light and water deficit. The stomata opened wider at first, then closed rapidly, so that they were fully closed shortly after photosynthesis stopped. Presumably, the latter is not controlled by the stomata in this case. If the leaf was not detached, the stomata closed only partially when placed in very dry air (v.p.d. = 38 mm. Hg). If the air was freed of carbon dioxide, the stomata

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opened considerably and remained constant for at least 1 to 2 hr. Photosynthesis measurements were not made with these attached leaves. Presumably, it was at a low level, especially with the carbon dioxide-free air. Many observations of photosynthesis made in the field (59, 60) indicate that over a wide range of soil moisture and relative humidity there was no effect on the photosynthesis of alfalfa. If the soil was allowed to become too dry, however, though wilting did not occur, photosynthesis was lowered but it returned to normal when the soil was moistened. Schneider & Childers (45) measured photosynthesis transpiration and respiration of potted apple trees and also trees in the field. When the moisture in the soil fell a little below field capacity the rate of photosynthesis increased significantly. Just before wilting was evident, the rate fell below normal—55 per cent in one case. After wilting the rate fell as much as 87 per cent. A tree showing slight wilting in the field had rates ranging from 20 to 70 per cent below normal. After watering, full photosynthetic function was not recovered for 2 to 7 days even though the leaves regained their turgor in a few hours. Transpiration rates usually went along with photosynthesis rates but respiration was increased during wilting; 60 per cent in one case.

Mitchell (35) measured the rate of photosynthesis of a number of plants at different relative humidities. At 26°C. there was no significant difference between the rates at 70 to 80 per cent, 40 to 50 per cent, and 10 to 20 per cent, even though, in the drier atmospheres, the stomata appeared to be closed. It was found, however, that both in moist air and in dry air, carbon dioxide absorption of *P. hortorum* increased to a maximum at 28° to 30°C., then fell off to about 50 per cent at 40°C. This effect was independent of moisture since there was no change in moisture content of the leaves. It represents the inhibiting effect of high temperatures on the photosynthetic process.

Temperature

The effects of temperature on photosynthesis are complex. The process consists of photochemical reactions that are not temperature-dependent, together with chemical reactions that have the usual Q_{10} value of about 2 or 3. Respiration also has a Q_{10} of about 2.0. The following examples illustrate Q_{10} values for photosynthesis which have been reported: Noddack & Kopp (37), *Chlorella* 2.07; Emerson (12, 13), *C. pyrenoidosa* 1.9 to 2.7, *C. vulgaris* and *Gigartina* 25.0 to 2.3; McAlister (34), wheat 1.0 to 1.9; Decker (10), pine trees 1.06 to 1.14; and Thomas & Hill (61) alfalfa 0.89 to 1.10. The high values with algae were found in media rich in carbon dioxide or bicarbonate; the values near unity involved normal air. In the latter case, carbon dioxide probably limits the reaction and the photochemical "light" processes control it. With high concentrations of carbon dioxide the "dark" reactions control, giving the temperature coefficients of ordinary chemical reactions (14). The very high Q_{10} value quoted for *C. vulgaris* was found at 1° to 6°C.

Apart from the direct effects on photosynthesis and respiration, temperature is known to affect the overall processes of assimilation which, in turn, may be reflected in a modification of the rate of photosynthesis. Some of these phenomena have been elucidated by studies conducted in the controlled environments of the Earhart Laboratory in Pasadena. Went (67, 68) described thermoperiodicity in the growth and fruiting of the tomato, using the growth rate of the stems as the criterion. Most of the elongation occurs at night, and is slight during bright days. On cloudy days there is less difference between day and night elongation. If the plants were transferred to the dark, growth continued for about 24 hr., then stopped rather abruptly. Conversely, if these plants were now illuminated, growth did not start for about 20 to 24 hr. The elongation therefore appeared to lag about one day behind the photosynthesis that it expressed. This time is required to transport sugars to the growing zones.

Went grew his tomato plants under conditions as nearly optimal as possible with respect to illumination and nutrient supply. The only other environmental factor which appeared to be critical was temperature. Plants maintained continuously at 26.5°C. grew well but fruited poorly. A steady growth rate of 23 mm. per day was maintained after the plants were 30 cm. tall and were kept trimmed to one stem. This rate could be increased to 27 mm. per day by maintaining a day temperature of 26.5°C. and a night temperature of 17° to 20°C. Fruiting was also most abundant under these conditions.

Assuming that the extra growth of 17 per cent also represented a corresponding increase in dry weight, it may be roughly calculated that the decrease in night temperature probably did not reduce the night respiration rate enough to conserve more than 8 to 10 per cent of the dry matter. It seems likely that the photosynthetic level of tomato plants grown at 26.5°C. during the day and at 17° to 20°C. at night was somewhat higher than the constant-temperature plants. Went pointed out that many other plants show this thermoperiodicity and that for maximum growth and best functioning of the plant, optimal day and night temperatures, usually within fairly narrow limits, need to be maintained.

In further studies, Went (68) concluded that while photosynthesis of tomatoes was only a little more rapid at 26°C. than at 18°C., it was the limiting reaction during the daytime for the overall processes that lead to growth. Sucrose in the leaves was clearly correlated with external conditions. It rose rapidly in the morning, decreased slightly in the afternoon, then fell to a low value at night. Considerably more sucrose was present at 26.5°C. than at 18° to 20°C. Translocation of sugar increased with decreasing temperature. Because of this, 18°C. is a more favorable night temperature than 26°C. Still lower temperatures are unfavorable because they reduce other reactions involved in growth. Elongation of excised stems and roots decreases regularly with decreasing temperatures. Stems on intact tomato plants have a maximum elongation rate at 18°C. The temperature effects on tobacco (6)

were similar to those on tomato except that both day and night temperatures could range from 20°C. to 26°C. without very definite effect.

The specific day and night temperatures for optimal growth vary over a wide range with different plants. This is forcefully illustrated by the work of Hiesey and co-workers (26, 28) on the grasses. In 15 grasses, including some crosses, optimal day temperature ranged from 10°C. to 18°C., and optimal night temperature from 3.5°C. to 16°C. In another group of 30 grasses, the optimal day temperatures ranged from 20°C. to 30°C. and the night temperatures from 6°C. to 16°C. These ranges define the habitats in which the different varieties and crosses will grow best, which has been confirmed by field tests at various stations in the West from the coast to high elevations. Dry weights of the plants indicate that the photosynthesis varies with the day and night temperature along with the vigor of the plants, being greatest with some plants at low temperatures and with others at high temperatures. For example, a *Poa ampla* plant gave a maximum yield of 125 gm. at 23°C. Day (D) and 10° or 14°C. Night (N) and a minimum yield to 41 to 60 gm. at 30°C. D. and 6°, 10°, or 17°C. N. A *P. compressa* plant gave 203 gm. and 114 to 117 gm. under the same conditions. A cross between these two plants gave 56 gm. and 18 to 28 gm. at these temperatures. Maximum yield of the cross was 68 gm. obtained with 20°C. D and 6°C. N. Another *P. ampla* plant gave a maximum yield of 115 gm. at 23°C. D and 6°C. N, and a minimum yield of 39 gm. at 30°C. D and 17°C. N. It was crossed with a *P. arida* which gave maximum and minimum yields of 140 and 67 gm. at these temperatures. The maximum yield of the cross was 100 gm. at 23°C. D and 14°C. N, and its minimum 45 gm. at either 20° or 30°C. D and 6°C. N. Evidently, parents that are ecologically similar can yield divergent offspring.

pH

The pH of the soil or nutrient solution generally affects the growth of plants indirectly by affecting the supply of nutrient elements to the roots. Toxic elements in some cases are made available which are ordinarily insoluble; for example, aluminum at low pH. Over a considerable range, however, pH usually has little effect on photosynthesis or growth. With water plants particularly, pH may affect photosynthesis directly by changing the amount of available bicarbonate ion and free carbon dioxide. Emerson & Green (13) observed years ago that the *Chlorellas* have the same photosynthesis in the range from pH 4.6 to 8.9. Respiration was 30 per cent higher at pH 4.6 than at 7.0 or 8.9. These were short-time experiments. Osterlind (38) aspirated *C. pyrenoidosa* suspensions with 0.1 per cent carbon dioxide and found that growth fell off linearly with decreasing pH. Since this plant cannot use bicarbonate ions and 0.1 per cent carbon dioxide is a limiting concentration, it appears that long exposures to the lower pH values reduced growth, which, of course, depends on both photosynthesis and respiration. The results of Emerson and Green and of Osterlind do not necessarily conflict since the exposure times differed widely. Steemann-Nielsen (54) showed that

the photosynthesis (oxygen evolution) of *Fontanalis dalecarlica* was unaffected by a pH of 3.0 for 1 or 2 hr. but after 20 to 23 hr. it was reduced by 75 to 80 per cent. *F. antipyretica* was somewhat more sensitive. It could not tolerate exposures below pH 4.0, and pH 3.0 stopped photosynthesis almost completely in 22 hr. In nature, the pH ranges of these two forms are 4.2 to 6.6. and 5.2 to 8.2, respectively. The former is never found in alkaline water, although it can tolerate an hour's exposure to pH 10.5; the latter is not found in the more acid waters. Plants that can utilize both bicarbonate ions and dissolved carbon dioxide are less circumscribed. *Scenedesmus quadricauda* (39) shows only slight growth at pH 5.5 and optimum growth at 6.5. Growth rate is directly proportional to the concentration of HCO_3^- below 10 μM. per liter. *M. spicatum* has a pH range from 4 to 10 in nature. Flowering water plants generally have a wider range of pH tolerance than the green algae.

It has been observed (54) that the alkalinity of the surface water of a lake in Denmark can be raised so high (above pH 10) by the absorption of HCO_3^- ions and release of OH^- ions in photosynthesis that algae near the surface are killed before evening. The pH is reduced and a fresh supply of algae is brought up from below by vertical mixing during the night. In the summer the algal population of the lake is said to be definitely limited by this pH effect.

Mineral Nutrition

No attempt will be made at this time to review in detail the voluminous literature bearing on the subject of the effect of mineral deficiencies on photosynthesis. In general, it is probably true that the effects are indirect by reducing the vigor of the leaves and interfering with the dark reactions of metabolism. Sulfur deficiency (61) and nitrogen deficiency (33) cause varying degrees of chlorosis which might interfere more directly by reducing the chlorophyll content of cells. The same may be true of other deficiencies, e.g., iron, manganese, etc. Thomas & Hill (61) observed that sulfur-deficient sugar beets gave no increase in photosynthesis when the air was enriched with carbon dioxide. Presumably, the photosynthetic apparatus was fully taxed with normal air and there was no excess capacity to handle more carbon dioxide. Sulfur deficiency lowered the photosynthetic level of alfalfa, tomatoes, and sugar beets but did not affect respiration appreciably. Gregory & Richards (18) found subnormal assimilation rates in barley leaves that were deficient in various nutrients. Potassium deficiency was complicated by an increased respiration rate while phosphorus deficiency produced decreased respiration. The assimilation rates would need to be adjusted for these differences in respiration to give a true relative measure of the photosynthesis.

Loustalot, Gilbert & Drosdoff (33) studied the effect of nitrogen and potassium levels in tung seedlings on the growth, apparent photosynthesis, and carbohydrate composition of the plants. Low, medium, and high levels of both elements were used in all combinations in the nutrient solutions. The nitrogen in the leaves ranged from 1.4 to 2.6 per cent, the potassium

from 0.5 to 1.4 per cent. The authors state that there was no statistical interaction between the nitrogen and the potasium. Therefore, they present the dry weight and photosynthesis data for each element, independently of the other.

Employing seedlings which were started in complete nutrient solutions, growth as measured by the increase in dry weight of the plants was rapid and nearly identical at the two higher levels of nitrogen and potassium. However, the concentrations of both elements in the leaves fell off gradually at the intermediate nutrient concentrations. It was noted that a slight chlorosis developed and photosynthesis began to decrease when the nitrogen level fell to 2.0 per cent about two weeks before the end of the experiment. At the lowest nitrogen level there was an overall decrease in growth amounting to about 25 per cent of the dry weight. Chlorotic symptoms began to appear about two weeks after the experiment started, at which time the nitrogen level was 2.0 per cent and there was a reduction in photosynthesis in the afternoons. Thereafter, photosynthesis fell sharply. Toward the end of the experiment, elongation ceased and many leaves abscissed. At this time the nitrogen content of the leaves had fallen to 1.4 to 1.6 per cent and rate of photosynthesis was reduced by about 65 per cent of the values with adequate nitrogen.

Potassium deficiency had a much smaller effect than nitrogen deficiency and was not significant when the leaves contained 0.8 per cent potassium or more. No deficiency symptoms were observed beyond retardation of growth. When the potassium level had fallen to about 0.5 per cent, overall dry weight was reduced by 25 per cent and photosynthesis by 35 per cent. Carbohydrate analyses showed a few significant changes associated with potassium deficiency, the principal ones being a decrease in nonreducing sugars and an increase in reducing sugars in the leaves and roots. Nitrogen deficiency caused similar but more significant changes in the leaves.

Schwabe (46) studied the mineral nutrition of bracken and found the effects of phosphorus and potassium supply on leaf production, total dry weight, leaf area, net assimilation rate, starch, and water content of the sporophyte. Plants were grown at two levels of phosphorus supply (1.0 and 0.1 standard) and three levels pf potassium (1.0, 0.11, and 0.012 standard) in three types of solution in which the cations were mainly sodium (A), calcium (C), or ammonium (M).

Deficiency of either potassium or phosphorus alone strongly depressed the total dry weight. The ratios of the high and low phosphorus dry weights at the three potassium levels were as follows:

Yield	High K	Med. K	Low K
High P/Low P	6.1	1.9	1.1

Evidently phosphorus deficiency decreased the yield of dry matter sixfold at the high potassium level (1.85 gm. potassium per pot); twofold at the medium level; and only slightly at the lowest level.

The high calcium solution (C) invariably gave better growth than either of the other two. Solution M was better than A when the phosphorus level was high. The reverse was true with phosphorus deficiency. At final harvest the dry weights in M and A were 83 and 58 per cent, respectively, of those in C. The potassium levels in solution C gave ratios of yields, 1.5 for high K/medium K and 7.5 for high K/low K. Similar ratios were obtained for the other solutions.

In a limited experiment with nitrogen and phosphorus in which plant levels of each element were set at 1.0, 0.11, and 0.012 of an adequate supply it was found that the lowest nitrogen level caused early death of the plants while at the intermediate level the plants continued to grow but the leaves were rather yellow, hard, brittle, and few in number. The lowest phosphorus level caused many leaves to die and at harvest left only a few living leaves of very small size. Dry weights at intermediate phosphorus and nitrogen levels were about 30 per cent of the high levels. Lowest phosphorus gave 5 per cent and lowest nitrogen 0.6 per cent of the yields with adequate nutrients.

Schwabe (46) showed that leaf area and net assimilation parallel the dry weight effects so that the latter is a fair expression of the photosynthetic processes. Carbohydrate and moisture values served to explain some of the effects noted. The data for bracken were compared with corresponding data obtained earlier for barley and flax by other investigators (18, 42, 47) and the conclusion was drawn that the three crops respond similarly to nitrogen, potassium, and phosphorus deficiencies.

Effect of Age

Singh & Lal (50), working with leaves of flax, sugar cane, and wheat, found that the rates of photosynthesis, which were slow for young immature leaves, increased to a maximum as the leaves matured, then decreased with increasing age. *Chlorella* cells likewise are known to increase in activity for the first few days and later to decrease in activity with increasing age. For this reason the age of the cells is carefully controlled in most experimental work.

Clendening & Gorham (8), employing isolated chloroplasts in the Hill reaction, found that the activity of these cells increased as the spinach or wheat leaves from which they were obtained enlarged and matured, then decreased in activity as the leaves grew older. Chloroplasts of young leaves suspended in the cell sap of old leaves and vice versa, gave the same pattern of activity. It was concluded that the aging effect was due to changes in the chloroplasts themselves.

Wilson (69) studied the effect of some environmental factors on the movements of the guard cells of privet and camellia. He found that assimilation and percentage of stomatal aperture gave closely similar curves for the leaves of the current year, but the older leaves gave a poor comparison, with the photosynthesis curves much lower, and often of a different shape, than

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the stomatal curves. Evidently, the photosynthetic efficiency of the leaves falls off with age. A similar observation was made by Freeland (16) on some conifers. The photosynthesis of individual detached clumps of needles each representing three years for six species of pine, fir, and spruce, was measured under controlled conditions. The variously aged needles were run in parallel experiments. Maximum activity was observed at the time the needles reached apparent maturity during their first year. During the second and third years the rate of photosynthesis decreased slowly with age, the percentage decrease of carbon dioxide absorption in 100 needles between the first and third years ranging from 30 to 90 per cent. Western yellow pine showed the largest decrease, Scotch pine, white pine, and spruce the smallest. Because some needles tend to increase in weight with age, these percentage changes would be greater if calculated on a weight basis. It should be pointed out that Freeland's results do not agree with earlier results by Stälfelt (52), who found that the activity of spruce needles increased for five years and pine for at least three years before declining. The increased activity was most marked when the trees were illuminated with 30 per cent of full sunlight.

The reason for the discrepancy between Freeland's and Stälfelt's findings is not apparent. Freeland notes that his measurements were made under uniform controlled conditions of light, temperature, and carbon dioxide concentration, and that the different aged needles were run at the same time, whereas Stälfelt did not have this control. It was also suggested that the soot and dirt on the older leaves might have caused the discrepancy by interfering with photosynthesis. However, washing the leaves did not change the picture. Freeland's trees grew near Northwestern University at Evanston, Illinois, where there is considerable air pollution. The condition of Stälfelt's vegetation is not stated. However, in view of the observations of Bleasdale (4) on coal smoke and of Koritz & Went (32) on smog, it is possible that the activity of the needles at Northwestern was reduced by such pollutants in the atmosphere apart from the solid deposit on the leaves.

Efficiency of Photosynthesis

It is well known that the overall efficiency of photosynthesis in the field does not exceed 2 per cent. This is radically less than the maximum efficiencies which have been measured for *Chlorella*, whether one accepts the nearly perfect efficiencies of Warburg at very low light intensities or the more generally observed values to 8 to 10 quanta per molecule of carbon dioxide representing efficiencies of about 20 to 25 per cent in white light.

A low photosynthetic efficiency for land plants in the field as compared with the algae under optimum conditions is inevitable. First, light saturation in the field occurs with about one-half to one-fourth of full sunlight intensity, resulting in nonutilization of one-half to three-fourths of the incident energy. Second, under optimum conditions, light saturation in the algae occurs at a lower carbon dioxide concentration than in the higher plants. Since the latter, under field conditions, are limited to a concentration of 300 p.p.m. carbon

dioxide their full photosynthetic capacity is never attained in sunlight. These two considerations alone may reduce the photosynthetic efficiency in the field by a factor of at least 6 or 8 below the values observed for algae. Other considerations, like moisture supply and nutritional level, also effect the utilization of light in the field.

In an effort to elucidate the metabolic reactions associated with photosynthesis, and the environmental factors producing different chemical trends, Spoehr & Milner (51) made elementary analyses of *Chlorella* grown in various environments and found that the ash-free composition of the cells varied markedly in different media and with different conditions of growth. Some were high in oxygen and approached the composition of carbohydrates, others were low in oxygen and resembled the lipids. Still others were intermediate, resembling proteins. Nitrogen varied with the supply in the nutrient solution.

The authors multiplied the percentage of carbon by the ratio of oxygen to carbon in carbon dioxide (viz., 2.664) and the percentage of hydrogen by the ratio of oxygen to hydrogen in water (7.936), added these values together and subtracted the percentage of oxygen to obtain a number which expresses the state of reduction of the compound. Nitrogen was ignored in this calculation. The result was then figured as a percentage of the value for methane (398.7) which is asssumed to have maximum reducing power. This final percentage was designated the "R-value" for the cells. It has been pointed out by Kok (31) that the R-value theoretically represents 7.6 times the heat of combustion in kcal. per gram.

With these data and the percentage of nitrogen, it is possible to calculate the percentages of carbohydrates, C, proteins, P, and lipids, L, in the mixture. These have R-values as follows: C = 28, P = 42, and L = 67.5. Protein is obtained as N × 6.25; the others by solution of simple algebraic equations. Application of this method to a number of cell preparations was satisfactorily checked by direct chemical analysis of the cells for carbohydrate, protein, and lipid.

With 5 per cent carbon dioxide in air, the yield at 200 watts and 15 days was 10 times as great as with normal air but only 78 per cent of the yield with 5 per cent carbon dioxide in nitrogen. The R-values were 51.4, 40.4, and 55.2, respectively. At 100 watts and 11 days, 5 per cent carbon dioxide in nitrogen gave the same yield as 5 per cent carbon dioxide in air, but 5 per cent carbon dioxide in oxygen gave much poorer plants. Excellent growth was obtained without any oxygen in the gas whereas, according to Emerson (14), some oxygen is necessary at least for certain algae. Light in excess of 300 watts or a carbon dioxide concentration of 10 per cent reduced growth. Intermittent light (12 hr. on and 12 hr. off) gave the same result as the equivalent continuous illumination using 5 per cent carbon dioxide in nitrogen, but 8 to 25 per cent better growth using 5 per cent carbon dioxide in air.

The R-value was increased by lowering the nitrogen concentration of the nutrient solution. At 0.0004 *M* nitrogen, the R-value was 57 or more; at

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0.001 *M*, it did not exceed 47. At 0.02 *M*, the cells were enlarged and dry matter and R-values were low. Best results were obtained by starting with 0.00225 *M* nitrogen in the nutrient solution and allowing the concentration to fall off with time, thus producing rapid initial growth and high R-values.

Optimum temperature was 20° to 25°C. Growth fell off at a higher and lower temperatures and was zero at 10° and 40°C. In some systems there was no growth at 30°C. While it was not suggested that the fundamental reactions of photosynthesis were affected by the different environmental factors, the overall reactions of assimilation, including the yield and composition of the plants, were evidently affected profoundly.

Kok (31) has extended the procedure of Spoehr and Milner by measuring the absorbed radiation, the rates of photosynthesis and respiration, and the heat of combustion in addition to the elementary composition and increase in dry weight of *Chlorella* cells. The efficiency of photosynthesis could be calculated from these data as the ratio of the energy in the cells to the absorbed energy. Values ranging from 6 to 24 per cent were found for the percentage of absorbed energy that could be accounted for in the vegetation produced. The lower values were found with less vigorous strains of *Chlorella* which had protracted light exposures in nitrogen-deficient media; the highest values were obtained using the most active strains in complete nutrient solutions, with short or discontinuous exposures to light. The latter cells were rich in protein, as were also cells grown out-of-doors that were subjected to sunlight and night conditions. In general, it was observed that with prolonged illumination and progressively higher light intensities, the percentage of protein in the cells decreased and the carbohydrates and lipids increased. Rest periods in the dark reversed these trends if adequate nitrogen was present. Kok suggested that at

> "high light intensities, carbon fixation outranges nitrogen fixation, and cellular composition shifts to low protein content. After prolonged exposure, cell division rate and photosynthetic efficiency may decrease. Upon darkening . . . protein synthesis and cell division restore cellular composition and size. Probably natural days and nights are of primary importance for the efficient utilization of . . . sunlight."

Toxic Gases

Photosynthesis has been used as a yardstick for evaluating the effect of fumigation of plants with toxic gases. For this purpose, it is an ideal tool, because it is sensitive to most of the fundamental activities of the plant, and its measurement can be carried out on a continuous schedule without interfering with the normal functioning of the plant. If the photosynthesis is unaffected by a fumigation treatment, it is unlikely that the plant is injured. Conversely, reduction of photosynthesis is a quantitative measure of the extent of plant damage.

Earlier work with sulfur dioxide (29, 59) showed that sublethal concentrations of sulfur dioxide could be tolerated for long periods of time without interfering with carbon dioxide uptake. Somewhat higher concentrations

caused a sharp inhibition of assimilation, but if the fumigation was stopped before permanent injury to the leaves occurred, a normal, or slightly higher than normal, photosynthetic level was re-established. This usually required about 1 to 2 hr. which was about the time needed to oxidize accumulated sulfite in the cells. Such treatments resulted in no significant reduction in yield of the plant. Still higher concentrations caused acute leaf injury, which reduced photosynthesis permanently in proportion to the leaf area destroyed. "Chronically" injured areas of cotton leaves appeared to retain about half their photosynthetic capacity.

More recent work with hydrogen fluoride (58) gave a somewhat modified picture from that of sulfur dioxide. Again, sublethal concentrations could be tolerated for long periods without interference with assimilation. However, in most species there was ready translocation of the absorbed fluoride to the tips or margins of the leaves, where toxic concentrations were built up, causing necrotic areas. Photosynthesis was reduced by the extent of these injured areas, but the green portions of the leaf remained fully functional. The range of "sublethal" concentrations with different species is very wide. In gladiolus, as much as about 10 p.p.b. in many 6- to 8-hour daily fumigations could be disposed of by translocation to permit the green areas to function fully. In cotton, 400 to 500 p.p.b. could be similarly tolerated. Other species had intermediate values. Higher concentrations than these thresholds caused a reduction in photosynthesis in excess of apparent leaf injury. Recovery from this inhibition was much slower with hydrogen fluoride than with sulfur dioxide. Recovery was rapid at first, then gradually approached the end value, requiring a few hours to 2 to 3 weeks for complete recovery. This may be equivalent to complete inhibition of photosynthesis for as long as 2 to 3 days. The temporary reduction of photosynthesis may be considered "invisible injury." Its temporary character needs to be emphasized because the photosynthetic function was eventually completely restored except to those areas which showed permanent visible damage. The slow recovery from concentrations of hydrogen fluoride that interfere with photosynthesis, as compared with sulfur dioxide, is due to the fact that, whereas it is only necessary to oxidize sulfite to sulfate in the leaf to remove most of the sulfur dioxide toxicity, an excessive concentration of fluoride must be removed by translocation, and possibly also by volatilization or the formation of less toxic organic compounds. All of the latter processes are rather slow.

Studies on smog suggest that it may reduce the growth rate of plants without causing visible damage to the leaves (32). Closure of the stomata has been observed but photosynthesis measurements have not been made. It is apparent that the observed reduction of the growth rate can only mean reduced photosynthesis. This is another possible example of "invisible injury." As already suggested, this type of damage may also occur in industrial areas where coal smoke is an important air pollutant, since it has been found (4) that the growth rate of grasses may be reduced without visible injury to the leaves.

Freeland (15) sprayed beanplants with solutions of 100 p.p.m. 2,4-

dichlorophenoxyacetic acid (2,4-D), indole butyric acid (IBA), indole acetic acid (IAA), *para*-chlorophenoxyacetic acid (CPA), and naphthoxyacetic acid (NAA) until the plants were nearly dripping wet. NAA stimulated photosynthesis about 10 per cent. The other compounds reduced photosynthesis and the level fell lower each day for 2 to 4 days. Reduction due to 2,4-D was about 20 per cent; IBA, 20 per cent; IAA, 70 per cent; and CPA 90 per cent. Wedding *et al.* (66) found that inhibition of the photosynthesis of navel orange leaves and of *C. pyrenoidosa* by 2,4-D was due to the undissociated molecules of the compound at concentrations as low as 2×10^{-7} M in solutions of total 2,4-D concentration $2\times10^{-3}M$.

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CHEMICAL CONSTITUTION AS RELATED TO GROWTH REGULATOR ACTION[1]

By Robert M. Muir
Department of Botany, State University of Iowa, Iowa City, Iowa
and Corwin Hansch
Department of Chemistry, Pomona College, Claremont, California

Introduction

Recent volumes of this series have included excellent reviews of the progress and new interpretations in the field of growth regulators, particularly the auxins [Bonner & Bandurski (11), Gordon (22), van Overbeek (43) Veldstra (60)]. A survey and appraisal of the publications which have appeared since these reviews were prepared would have little value. Although Veldstra's review of the literature concerning the significance of chemical constitution in the action of growth regulators is as detailed and analytical as might be desired, it is essentially an interpretation of the available data in terms of a physicochemical mode of action of regulators with the reservation that proof of the supposition must await the identification of the reactive entity within the cell. No such identification has been accomplished and this review will not attempt to resolve the question of physicochemical activity versus chemical reactivity. Rather, it will consider certain aspects of the general problem of analysis of chemical constitution and activity together with the experimental facts which an hypothesis relating constitution and activity must explain.

Analysis of Activity of Regulators

Plant tissue and response.—The principal problem of an appraisal of all the literature pertaining to the chemical constitution of plant growth regulators lies in the correlating and reconciling of data derived from many different biological responses such as the formation of roots on stem cuttings, induction of parthenocarpic fruit development, epinasty of leaves, inhibition of root growth, elongation of coleoptile and epicotyl tissue, and reversal of curvature resulting from tissue tension in slit epicotyls (pea test) and coleoptiles. An attempt to correlate and reconcile all such data must assume that these growth responses in all plants are controlled by the same hormone, indoleacetic acid, having the same mode of action in each response.

Such an assumption was unwarranted when auxin *a* and auxin *b* were considered to be growth hormones (70), and is subject to question now because of the isolation of indoleacetonitrile from plant tissues (26) as well as the evidence of unknown growth hormones obtained by paper chromatog-

[1] The survey of the literature pertaining to this review was concluded in November, 1954.

raphy (7, 54). Yet the assumption is fundamental in the suggestion that the activity of a compound, as measured in different responses, may reflect the relative penetration of the compound to reactive sites or other unknown properties of the compound (65), and that the relative activities of different compounds in a single response are unreliable measures of effect in the common growth reaction. The same assumption is responsible for the interpretation of the phenomenon wherein a compound which induces a response in the tissue of one species but not in that of another, must have been converted in the former to a structure similar to those which cause the same or similar response in both types of tissue (43, 57). The assumption appears to be involved also in the explanation of effects of compounds which do not follow the pattern of relationship of activity and concentration shown by other regulators and are therefore regarded as having an unknown interaction (synergistic action) with the hormone rather than a direct effect similar to that of the hormone (5).

There are numerous instances in which we are unable to reconcile data from different plant responses for the same molecular structures and, in some cases, the most logical interpretation questions the above assumption. In fact, Audus (6) has suggested that the effects of various regulators on the growth of roots can be interpreted more logically in terms of control by a native inhibitor rather than by indoleacetic acid.

Recent studies of molecular structure in the action of regulators for the most part have employed either the straight growth of segments of *Avena* coleoptiles, or curvature of slit epicotyls of pea (pea test), or both tests for growth activity. For many compounds the data of these tests cannot be reconciled (39, 56, 60, 65). The complex nature of the pea test and the participation of large numbers of wounded cells as well as other features make it less certainly a strict test of effects of growth regulators on the elongation process, yet we may only conjecture reasons for the differences between the two test methods. In this connection and also in the general consideration of growth in the pea, the recent discovery of another nitrile compound in plant tissue may have considerable significance.

The first nitrile to be isolated from plant tissue and found to have growth effects was indoleacetonitrile (26) which is more effective as an auxin for *Avena* coleoptile tissue than indoleacetic acid (9). It was isolated from cabbage and since has been reported from other tissues (7). The discovery of the second nitrile with growth effects came about through the investigation of the substance in the seeds of *Lathyrus odoratus* which causes the skeletal abnormalities characteristic of lathyrism in man and domestic animals. Schilling & Strong (47) and Dasler (15) have isolated and identified a crystalline substance which causes the skeletal changes in rats and found it to be β-(γ-L-glutamyl)-aminopropionitrile. Although a diet of seeds of *Pisum sativum* does not give symptoms of lathyrism in the rat, the addition of a water-soluble extract of the seeds does produce the symptoms in the monkey (53). Preliminary investigations of the activity of this

substance in the straight growth of coleoptile sections indicate that it is an antagonist toward indoleacetic acid at low concentrations and that the antagonism is readily overcome with higher concentrations of indoleacetic acid (37). Similar results were obtained with aqueous extracts of seeds of sweet pea and garden pea. Thus, it appears that the investigation of nitrile metabolism in plants may have an important bearing on the control of growth by hormones.

Stowe & Thimann (54) have reported that indoleacetic acid can be detected by paper chromatography in solutions of indoleacetonitrile on which *Avena* coleoptile sections grow for 24 hr. as well as in extracts of the coleoptile tissue, thus indicating that the tissue converts the nitrile to the acid. Thimann (57) has also reported that although the nitrile has no activity in the pea test, both the pressed sap and the ether extract of sections of coleoptiles grown for 48 hr. on a solution of the nitrile cause sufficient response in the pea test to indicate a conversion of more than 50 per cent of the nitrile by the coleoptile tissue.

Another type of metabolic transformation of growth regulators which has been the subject of recent study is β-oxidation of long side chains. Synerholm & Zimmerman (55) suggested such a transformation leading to the active acetic acid homologue to explain their observation that in a series of ω-2,4-dichlorophenoxyalkylcarboxylic acids only those with an even number of carbon atoms in the side chain caused epinasty in tomato plants. Fawcett, Ingram & Wain (16) have studied the oxidative degradation of phenoxy acids with varying lengths of side chain within the tissues of the flax plant and have obtained evidence that β-oxidation does, in fact, occur. Such acids with an odd number of carbon atoms in the side chain gave rise to appreciable quantities of phenol while those with an even number yielded only traces. In this investigation the degradation product of acids with an even number of carbon atoms in the side chain would be phenoxyacetic acid and no activity was displayed in epinasty or elongation of *Avena* coleoptile sections, yet in the pea test, acids with an odd number were active and those with an even number were inactive. Fawcett, Ingram & Wain suggest that in the tissue of the pea a specific intermediate product in the degradation of the side chain is responsible for the difference in response.

Further study of the growth-regulating activity of 4-chloro-, 2,4-dichloro-, and 2,4,5-trichloro-phenoxy acids in relation to their β-oxidation has been made by Wain & Wightman (68). In the 4-chloro- and 2,4-dichloro series the acids with even numbers of carbon atoms in the side chain which would yield active acetic derivatives caused elongation of sections of wheat coleoptiles, epinasty of tomato leaves, and curvature in the pea test, whereas the acids with odd numbers of carbon atoms were inactive. In the 2,4,5-trichloro series, a similar relationship of chemical constitution and effect was noted in the elongation of the coleoptile tissue. However, only the acetic acid caused an effect in the epinasty and pea tests, thus indicating a lack of conversion in the pea and tomato which Wain & Wightman suggest may be

due to the effect of nuclear substituents in the β-oxidation mechanism or an effect on the penetration and movement within the tissue. When the pea test was made with solutions on which coleoptile tissue had grown for 72 hr. the response was the same as for coleoptile tissue, indicating the conversion of the higher homologues to the acetic derivative. The conversion of the butyric acid derivative was demonstrated by paper chromotography.

Kinetic analysis.—The most important contribution to the analysis of activity of growth regulators in recent years has been the discovery of McRae, Bonner & Foster (19, 32, 33, 34) that the elongation of *Avena* coleoptile sections induced by exogenous regulators may be described and analyzed by the methods of study of enzyme kinetics proposed by Michaelis and Menten. This discovery provides a means of determining the nature and properties of the reactive entity within the plant cell. The kinetic analysis of elongation has already given precise explanations of the inhibition of growth by high concentrations of auxin (19), the antiauxin effect of molecular structures similar to auxins (32, 33), and the interaction of chemically different auxins (34). The possibility of determining the binding affinities between regulators and the reactive entity within the cell as reported by Foster (18) affords the first critical approach to the question of chemical activity as opposed to physicochemical activity.

These investigators employ a kinetic treatment according to the basic equation

$$\mathrm{E} + \mathrm{S} \underset{}{\overset{K_S}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k} \mathrm{E} + \text{Products} \qquad 1.$$

widely used in enzyme kinetics (20). In this equation E represents an enzyme system in the coleoptile section, S the growth regulator, ES the active complex which is the growth limiting factor under the experimental conditions, K_S the dissociation constant of the complex ES, and k the velocity constant relating concentration of ES to product formation which is growth in this application. This equation may be expressed as

$$v = \frac{V_{\max}[\mathrm{S}]}{K_S + [\mathrm{S}]} \qquad 2.$$

where $V_{\max}$ is the maximum growth rate obtained when sufficient regulator, S, is present to saturate all enzyme molecules, E. In the above equation v is the reaction velocity (growth rate of the coleoptile section) and K_S and $V_{\max}$ are constants. Writing the equation as its reciprocal we obtain

$$\frac{1}{v} = \frac{K_S}{V_{\max}[\mathrm{S}]} + \frac{1}{V_{\max}} \qquad 3.$$

Plotting $1/v$ against $1/[\mathrm{S}]$ would thus give a straight line and the data for growth rate as related to concentration of auxin obtained by McRae, Foster & Bonner (34), when so plotted, do give a straight line (Fig. 1) thus confirming the assumption that the auxin reacts with a plant substrate according to equation 1.

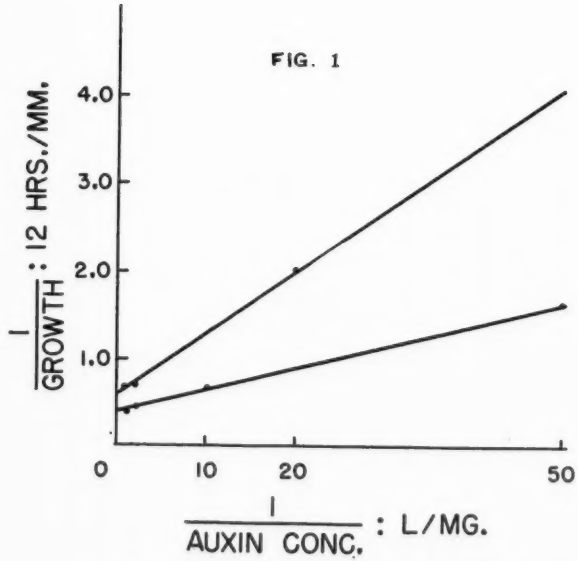


FIG. 1. The reciprocal of growth rate of *Avena* coleoptile sections plotted as a reciprocal function of the concentration of 2,4-dichlorophenoxyacetic acid (upper line) and indoleacetic acid (lower line) [after McRae, Foster & Bonner (34)].

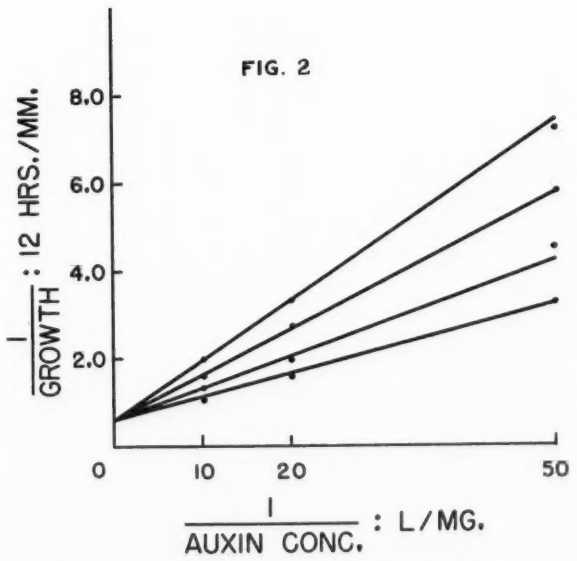


FIG. 2. The reciprocal of growth rate of *Avena* coleoptile sections plotted as a reciprocal function of the concentration of 2,4-dichlorophenoxyacetic acid alone (bottom line) and in the presence of 0.1 mg./l., 0.5 mg./l., and 1.0 mg./l. of 2,6-dichlorophenoxyacetic acid (upper lines in order of ascent) [after McRae & Bonner (32)].

Assuming that an inhibitor reacts with the same substrate or enzyme as does the auxin, equation 1 becomes

$$E + I \overset{K_I}{\rightleftarrows} EI \qquad 4.$$

where I represents the inhibitor, EI the inactive inhibitor-enzyme complex, and K_I the dissociation constant for the complex EI. When an inhibitor is present equation 3 becomes

$$\frac{1}{v} = \frac{1}{V_{max}}\left[K_S + \frac{K_S[I]}{K_I}\right]\frac{1}{[S]} + \frac{1}{V_{max}} \qquad 5.$$

Comparing equation 3 with equation 5 it is seen that when $1/v$ is plotted against 1/[S] the competitive inhibitor increases the slope of the resultant line by the factor $K_S[I]/K_I$. The intercept, $1/V_{max}$, does not change. The experimental data of McRae & Bonner (32, 33) pertaining to the effects of phenoxyisobutyric and diortho-substituted phenoxyacetic acids on elongation of *Avena* coleoptile sections conform with this kinetic formulation (Fig. 2) and establish the competitive action of these regulators and auxins. For the auxin 2,4-dichlorophenoxyacetic acid, the plotting of the reciprocal of growth rate against reciprocal of concentration in the absence and presence of 2,6-dichlorophenoxyacetic acid shows that with increasing concentration of the latter the slope of the line increases but all lines have a common intercept.

Foster, McRae & Bonner (19) have shown that inhibition of growth by auxins at higher concentrations would be expected if the auxin reacts by a two-point attachment mechanism. The active complex would have an auxin molecule attached at two points on the substrate; as the concentration of auxin increases, the probability of two auxin molecules attaching at the two substrate sites increases and the number of two-point attachments of one auxin molecule forming the active complex progressively diminishes. If the kinetic formulation of equation 2 is altered to include the possibility of formation of an inactive complex between the enzyme and substrate, Foster, McRae & Bonner obtain the expression

$$v = \frac{V_{ex}[S]}{K_S' + [S] + [S]^2/C} \qquad 6.$$

where V_{ex} (corresponding to V_{max}), K_S' and C are experimentally determinable constants and $[S]_2/C$ is a measure of the probability that a second molecule of auxin, S, will become attached to the substrate before the first has consummated its two-point attachment. The experimental data are described accurately by this equation (Fig. 3).

Recently the validity of this kinetic analysis of elongation has been challenged by Bennet-Clark & Kefford (8). They interpret their data to indicate that a linear relationship does not exist between elongation and time at most concentrations of auxin, and thus the measurement of elongation

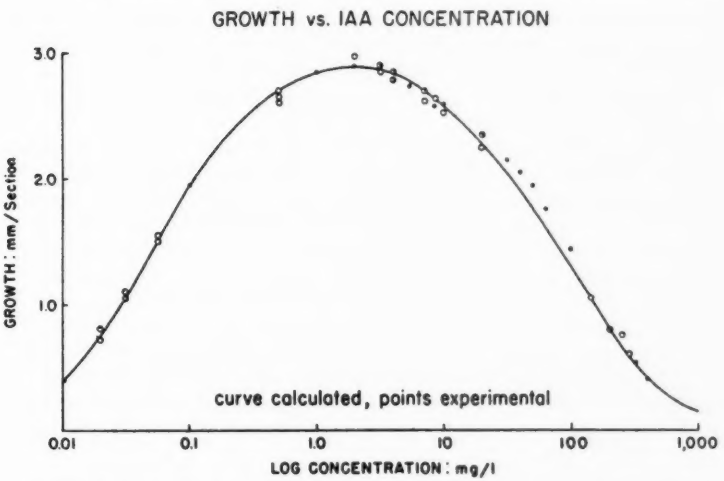


FIG. 3. Growth rate of *Avena* coleoptile sections plotted as a function of indoleacetic acid concentration. The solid line is the curve calculated from equation 6 and the individual points are data from five different experiments [after Foster, McRae & Bonner (32)].

after 12 hr. is not a measurement of the initial velocity of reaction as required for the kinetic analysis. A slightly sigmoid curve was claimed for low concentrations of indoleacetic acid suggesting a "lag phase." However, these data were obtained with coleoptile sections growing in 3 per cent sucrose without potassium maleate buffer at pH 4.5. Bonner & Foster (12) have shown that the data of Bennet-Clark & Kefford do, in fact, conform to a linear relationship within experimental error and that in the presence of the potassium maleate buffer the growth rate of coleoptile sections is constant over a wide range of concentrations of indoleacetic acid at time intervals of 10 min. to 20 hr.

The existence of a "lag phase" as envisioned by Bennet-Clark & Kefford has been demonstrated much more conclusively for 2,6-dichlorobenzoic acid, *ortho* chlorobenzoic acid, and 2,3,5-triiodobenzoic acid by Carroll (14). In these instances the "lag phase" appears logically explainable in terms of a slower reaction involving displacement of electron-attracting substituents by the nucleophilic substrate through an intermediate complex (23). It is not surprising that the precisely quantitative data required for the kinetic analysis of elongation are greatly dependent upon the experimental conditions. There is no more reason to believe that the exogenous sucrose supply constitutes a "normal" condition than that a balancing supply of potassium

does, and the interaction of these factors may be of fundamental importance in the growth reaction (14, 73). The pH of the medium in which growth of the sections occurs is also a very important factor. It appears that the pH of 4.5 selected by McRae & Bonner is the optimal condition for maximum growth (73).

In expressing the kinetic parameter, V_{max}, or maximum velocity of the growth reaction, as a constant, the ratio of the increment of growth to the original length gives a value independent of the initial length of the section which may be preferable to its expression in millimeters of increment (73). Ordinarily, the velocity values are obtained by the subtraction of growth occurring in the absence of the regulator from that in the presence of the regulator. Under certain experimental conditions and in the presence of certain regulators, the growth may be less than in the control medium as found by Ingestad (25). In this situation Ingestad proposes that the endogenous auxin concentration can be calculated and a value obtained for total auxin concentration. With the corrected concentration values, straight line reciprocal plots are obtained, and Ingestad suggests this result reflects the endogenous action of the regulators.

Use of buffer systems.—The pH of the medium may be of importance in the assay of weak acids for activity in the growth reaction of plant cells. The initial assay of the activity of 2,4,6-trimethylphenoxyacetic acid indicated a small but significant elongation effect of this compound (41). Subsequently, it was found that the elongation occurred as a result of the pH of some of the more concentrated solutions of the acid, for when the acid was adjusted to pH 5.6 no elongation effect was obtained, and low pH levels provided by hydrochloric acid alone were found to result in greater elongation than that which occurs in more alkaline media.

In testing such acids over a wide concentration range, the secondary effect of pH may be prevented either by adjusting the pH of the solution or by using a buffer system. The latter procedure, though seemingly the obvious choice, introduces additional problems for most buffers contain ions which may directly affect the growth of the tissue and the selection of the pH level for the system is in itself arbitrary. Simon & Beevers (49) have shown that as the pH of a solution of a weak acid is lowered to the pK of the acid the concentration of the acid required for a standard response becomes minimal. Since the pK values of the synthetic regulators show some variation, the use of a buffer system at uniform pH would not assay the maximum activity of all of the acids. Further, the pK values for many of the compounds would be at such a low pH as to be toxic to the tissue.

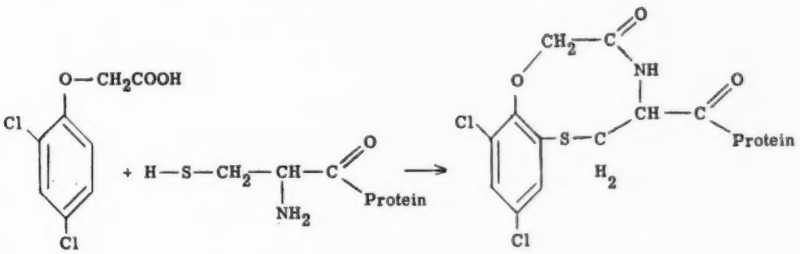
The most important difficulty in the use of buffer systems occurs in the testing of molecular structures possessing very slight activity in elongation. Low concentrations of phenoxyacetic acid have no effect on the growth of *Avena* coleoptile sections in water solutions. At concentrations of 2 or $3\times10^{-4}M$ adjusted to pH 5.6 the acid causes a small but significant growth

effect (39). Ingestad (25) found, however, that the acid at 1 and $3\times10^{-5}M$ in $10^{-3}M$ sodium citrate buffer of pH 4.5 inhibited the growth of coleoptile sections. A similar effect of the buffer system has been noted by Åberg & Khalil (5) on the antagonism of the (−) isomer of α-2,4,5-trichlorophenoxypropionic acid in which 10^{-5} and $10^{-6}M$ concentrations in Na_2HPO_4-KH_2PO_4 buffer at pH 5.9 caused inhibition of the growth of coleoptile sections. Smith, Wain & Wightman (52), however, found no inhibition of growth at concentrations of 0.01 to 20 p.p.m. in water solutions. There is no evidence to substantiate any interpretation other than an effect of the buffer system on the coleoptile tissue.

Regulators of the Aryloxy Acid Series

With the discovery of the growth-regulating properties of the chlorinated phenoxyacetic acids by Zimmerman & Hitchcock in 1942 (74), this group of analogues acquired fundamental significance in the analysis of chemical constitution as related to physiological activity. The study of the effects of substitution of halogens and methyl groups in the 2, 4, and 6 positions of the benzene ring revealed that substitution of both *ortho* positions caused complete loss of stimulatory effect on elongation (41). This observation was the basis for an hypothesis that the position on the benzene ring adjacent to the point of attachment of the side chain is directly involved in the growth reaction by a nucleophilic substitution together with the attachment of the carboxyl group of the side chain to a basic group (24).

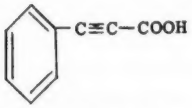
Such a two-point reaction is suggested to take place between the regulator and a cysteinyl unit of a protein as follows in Reaction I:



Reaction I

This hypothesis affords a very satisfactory explanation of the activity of *cis*-cinnamic acid in elongation and the inactivity of the *trans* form as well as other *cis-trans* acids of this type (24). If a growth regulator reacts at two points with two points of a plant substrate then a ring structure will result. It is a well-known fact (21) that cyclic structures containing a *trans* double

bond form with difficulty except in very large rings. Phenylpropiolic acid (see cut)



Phenylpropiolic acid

has been found to be inactive in the elongation of *Avena* coleoptile sections (40) and this is to be expected since rings containing triple bonds are highly strained unless they are very large (21).

Wain has reported (66) that 2,4-dichloro-6-fluoro- and 2,4-dibromo-6-fluoro-phenoxyacetic acids have high activity in the elongation of *Avena* coleoptile sections. In these compounds both *ortho* positions are substituted and the *ortho* reaction hypothesis does not explain their activity. Although more study of fluorine-substituted compounds is required to evaluate the significance of these results, it is possible that a reversible reaction at the *ortho* position, i.e., an adsorption reaction, would proceed with little more hindrance by a small fluorine atom than that by a hydrogen atom. If, however, the reaction is in the nature of the formation of a new covalent bond, then the F^- could be removed by a displacement mechanism. It has been shown (72) that fluorine is much more easily removed from an electron-rich benzene ring than are the other halogens. Wain has suggested that the reason for the activity of 2,4-dichloro-6-fluorophenoxyacetic acid compared with the inactivity of 2,4,6-trichlorophenoxyacetic acid is that in the first molecule the side chain may be free to rotate because of the small size of the fluorine atom, while the two large chlorine atoms in the second structure prevent rotation. However, this hypothesis fails to explain the activity of other regulators with both *ortho* positions substituted.

In 1951 Leaper & Bishop prepared all mono-, di-, and trichlorophenoxyacetic acids and tested them for activity in the inhibition of root growth, leaf epinasty, and initiation of roots on stems (27). Because of the inactivity of the 3,5-, 2,3,5-, and 3,4,5-acids in these tests they proposed that an open position *para* to the *ortho* position is required for activity and the active molecule is involved in the formation of quinoid compounds in plant cells. Wain & Wightman have examined these same compounds for activity in the straight growth of *Avena* coleoptiles and in the pea test (67). They find that in both tests the 2,3-, 2,3,4-, 2,3,5-, and 3,4,5-acids are active. Since these compounds do not have an open position *para* to the *ortho* position, the hypothesis of Leaper and Bishop is invalid. Further evidence against the hypothesis is the fact that 2,5-dihydroxyphenylacetic acid which should form a quinone readily is less active than phenylacetic acid; 4-chloroindoleacetic

acid, which is more active than indoleacetic acid, would require displacement of the chlorine for *para* quinone formation or destruction of the five-membered ring; and 2-methyl,5,7-dichloro-3-indoleacetic acid is active although all positions are blocked against quinone formation (39).

Thimann (56) has found that 2,6-dichlorophenoxyacetic acid has some activity in the pea test and proposes a novel electronic interpretation in which occupation of one or more of the *ortho* or *para* positions of the benzene ring results in the intensification of the nucleophilic influence at the remaining positions and occupation of all three positions prevents all reaction. The 3,5- acid would not have activation of the *ortho* or *para* positions and its inactivity would be explained. However, in this view the 2,6- compound should have activity approaching that of the 2,4-substituted ring which it does not, and alkyl substituents such as methyl groups should be very effective activators, which they are not. That 2,6-dichlorophenoxyacetic acid does not cause elongation of *Avena* coleoptiles has been reported by several workers (32, 39, 42, 65). The activity reported for the compound by Thimann has been suggested to be the result of the presence of active impurities (33). Wain, however, (65) states that activity of the compound in the pea test is dependent upon exposure of the pea seedlings to red light during growth.

This effect of red light may involve the formation of a reactive entity (receptor) within the cells as described by Liverman & Bonner (29), it may be due to the opening of the stomata allowing for high internal concentrations of the compound in the cortical tissue, or it may reflect some fundamental difference in the growth mechanism such as the participation of nitriles. In the pea test, it appears quite unlikely that activity dependent upon exposure to red light has any direct significance in the relationship of structure and activity. The fact that the greater part of our knowledge concerning chemical constitution and activity in plant growth can be applied to varied plant responses has caused undue emphasis to be placed upon conflicting data from different tests in the selection of a plausible interpretation. With more complete knowledge of the growth reaction in *Avena* coleoptile tissue, the differences in growth responses of other tissues may become valuable clues to the variation in the reaction among different tissues.

Recently an effect of the length of side chain on the activity of di-*ortho*-substituted phenoxy acids has been reported by Osborne *et al.* (42). Although the di-*ortho*-substituted phenoxyacetic acids were without effect in causing elongation of coleoptile sections, some activity was found for phenoxypropionic and phenoxybutyric acids with either chlorine atoms or methyl groups in both *ortho* positions. The composition of the medium in which the growth effects were observed was not described. The stimulatory effect on elongation was not obtained with similar compounds in which the 4-position was substituted (2,4,6-trichlorophenoxypropionic acid and 2-methyl-4,6,-dichlorophenoxypropionic acid) which suggests the possibility

of the di-*ortho*-substituted phenoxypropionic acids entering into the reaction at the *para* position when the *ortho* position is blocked.

In the study of the optical isomers of α-2-naphthoxypropionic acid, Smith & Wain (50) found the L-isomer to be inactive in the pea test and the elongation of *Avena* coleoptile sections while the D-isomer was active. Furthermore, 2-naphthoxyisobutyric acid was inactive in both tests. Although recognizing that the optical isomers may combine with an optically active cell constituent and thus form two compounds with different properties of which only one might have activity, they prefer an explanation involving three specific groupings of the molecule, the carboxyl group, the unsaturated ring, and an *alpha* hydrogen atom arranged around the asymmetric carbon atom, which must be stuitably placed for contact with the receptor groups. Thus, in the D-isomer these three groups are in the proper spatial arrangement and in the L-isomer they are not. The inactivity of the α-isobutyric acid is thus the result of its lack of the essential α-hydrogen atom.

Recently, an elaborate study by Wain and co-workers, employing six growth responses including the pea test and straight growth of coleoptile sections, has compared the activity of the acetic, α-propionic, α-butyric, and α-isobutyric side chains in substituted phenoxy acids and naphthoxy acids (17). In tests dependent strictly upon cell elongation the isobutyric acids were inactive, but in the pea test slight activity was shown by several isobutyric acids and high activity was shown by 2,4,5-trichlorophenoxyisobutyric acid. This observation may be regarded as additional evidence of the unique character of the response in the pea test. Wain and his coworkers have also shown that the inactive optical isomer is antagonistic toward the effect of the active isomer and that di-*alpha*-substituted phenoxyacetic acids have an antagonistic effect toward active acids (51, 52).

An extensive study of the effects of 19 pairs of optical isomers on the growth of flax roots has been made by Åberg (2). He interprets his data as indicating a similar characteristic of D-forms having auxin activity and L-forms being antiauxins except in the phenoxy compounds where the L-forms have neither auxin nor antiauxin properties. However, antiauxin properties are difficult to establish by this test since they are determined by the stimulation of root growth and such stimulation is invariably small for flax roots (1). Since Matell has been able to demonstrate that (+)-α-phenoxypropionic acid has the same configuration as D-(−)-α-alanine and ethyl D-(+)-lactate (30) and that the (+)forms of α-2-methyl-4-chlorophenoxypropionic acid and α-(2,4-dichlorophenoxy)-*n*-butyric acid have the D-configuration (31), it seems likely that all of the most active members of enantiomorphic pairs belong to the D-series.

Attractive as Wain's hypothesis may be for the explanation of the activity of one of two optical isomers and the requirement of an α-hydrogen atom, these characteristics of molecular structure may find explanation also in chemical reactivity. The difference in activity of optical isomers resulting

from an asymmetric carbon atom may be due to differences in reaction rates resulting from interference in combining with another asymmetrical center. Such interference has been shown in the slower reaction of the L form of mandelic acid with L-meththol in esterification compared with the rate of reaction of the D form of the acid (21). The effect of *alpha* substitutions in the side chain can be explained by the steroic hindrance of the carboxyl group (71); two alkyl groups on the α-carbon atom would so reduce the reactivity of the carboxyl group that the molecule would be without biological effect.

Certain observations are not in accord with the hypothesis of a three-point reaction. Phenoxyacetic acid with two α-hydrogen atoms should be more active than α-methylphenoxyacetic acid but the latter is much more active than the former. In this instance, a subsidiary effect of structure on penetration into cells may explain the difference in activity, for Mitchell and his co-workers have found remarkable movement of α-methoxyphenylactic acid and other *alpha*-substituted regulators within the plant (36, 46), and between plant roots. Molecular structures such as the substituted benzoic acids which have no α-hydrogen atoms should not induce elongation, yet, some do. Other compounds with activity such as *cis*-cinnamic acid do not have the three specific groupings in the same spatial relation as in the regulators with saturated side chains, and their precise fit to the receptor sites would not be possible. Finally, Wain's hypothesis does not explain the lack of growth-promoting activity for the di-*ortho*-substituted regulators.

Inherent in the concept of chemical constitution determining physiological activity is the interference in such action resulting from the presence of similar but not identical molecular structures. The study of such interference should yield equally significant data concerning the mechanism of the activity but interference, in contrast to the stimulation of growth, may be a nonspecific effect. McRae & Bonner (33) have pointed out that the only rigorous method for distinguishing between interference of a competitive nature and that arising from such effects as the destruction of reactive groups is the kinetic analysis formulated by Lineweaver and Burk.

In 1949, Bonner (10) described the antagonistic action of 2,4-dichloroanisole in the auxin effect. This observation was questioned by Audus & Shipton (6) but has been reaffirmed by McRae & Bonner (33). However, Thimann also regards 2,4-dichloroanisole as only a growth inhibitor and not an antiauxin (66). Competitive inhibition has been demonstrated for the di-*ortho*-substituted phenoxyacetic acids (32), 4-chloro- and 2,4-dichlorophenoxyisobutyric acids (33), phenoxyacetic acid, and α-methylphenoxyacetic acid (25) in the elongation of *Avena* coleoptile sections. Furthermore, McRae & Bonner (33) have demonstrated that 2,4,6-trichloroanisole which lacks a carboxyl group and, in addition, has both *ortho* positions blocked for reaction, functions neither as an auxin nor as an antiauxin. They have therefore suggested that those compounds reported as antiauxins may be classified

according to structure: (*a*) aryloxy compounds with a free *ortho* position but lacking a carboxyl group; (*b*) compounds with a carboxyl group but lacking a free (reactive) *ortho* position; (*c*) compounds with steric hindrance preventing reaction of the carboxyl group; (*d*) compounds with auxin activity but of low V_{max}; and (*e*) substituted benzoic acids lacking auxin activity. It appears that the most logical explanation of molecular structure in relation to competitive interference is furnished by the hypothesis of a two-point reaction between the growth-promoting regulator and the plant substrate involving the carboxyl group and an *ortho* position.

Similar difficulties are encountered in attempting to relate chemical constitution to antiauxin activity in different plant responses as are found in relating constitution to auxin activity. Åberg (1) found that 2,4-dichloroanisole and other α-naphthyl and 2,4-dichlorophenoxy derivatives were without conspicuous antagonism against the inhibition of growth of flax roots by 2,4-dichlorophenoxyacetic acid. He concluded that a carboxyl group was essential for attachment of the auxin antagonist. However, in a study of the effect of monoalkyl substituents other than methyl groups on the α-carbon atom of aryl- and aryloxyacetic acids it was found that the steric hindrance preventing reaction of the carboxyl group gave rise to antagonistic action (3). The antagonism of the latter compounds can best be explained by incomplete reaction of the carboxyl group (salt formation without subsequent amidization).

Åberg (4) has also found that molecules, in which the *ortho* position is blocked for reaction such as the 2,6-dichloro- and 2,4,6-trichlorophenoxyacetic acids, have an antagonistic action against the inhibition of root growth resulting from 2,4-D and that substitution of large alkyl groups in the *ortho* or *para* positions of phenoxyacetic and phenoxypropionic acids increases the antagonism of such molecules. The presence of chlorine in 2-isopropyl-4-chloro-5-methylphenoxyacetic acid significantly increased the antagonism as was found for 2,4,6-trichlorophenoxyacetic acid in comparison with the 2,6-dichloro acid.

Growth Regulators of the Substituted Benzoic Acid Series

Since the discovery by Bentley of the activity of 2,3,6-trichlorobenzoic acid in the elongation of *Avena* coleoptile sections, the substituted benzoic acids have been of considerable interest with respect to the relationship of chemical constitution and growth-regulator activity. In a study of a selected series of *ortho*-substituted benzoic acids, Muir & Hansch (38) found that those with any stimulatory effect upon elongation contained an electron-attracting group (halogen or nitro group) in the *ortho* position. On the basis of this common structural feature and the well-established electronic theory of organic reactions, it was suggested that the reaction of these compounds involved the displacement of the electron-attracting group whereas in the phenoxyacetic acid derivatives the reaction occurs by hydrogen displacement.

Hansch *et al.* (24) were able to demonstrate the release of chloride from 2,6-dichlorobenzoic acids during the growth of *Avena* coleoptile sections, thus substantiating the hypothesis.

Minarik *et al.* (35) have reported the results of tests of more than 200 substituted benzoic acids on the growth of cucumber roots and the responses of the bean plants. All but three of the 30 compounds listed by them as active in causing responses of bean plants have a halogen atom in the *ortho* position. Furthermore, of 35 compounds found to stimulate elongation of cucumber roots none contained a halogen atom in the *ortho* position and the most active were 3-nitro-4-halogenbenzoic acids of which 3-nitro-4-fluorobenzoic acid was shown to antagonize the inhibition of elongation induced by 2,4-D.

The hypothesis of a reaction involving the displacement of the *ortho* halogen atom in the substituted benzoic acids does not account for the slight activity of 2,6-dimethylbenzoic acid or the lack of activity of 2,4-dichlorobenzoic acid in the elongation of *Avena* coleoptile sections (38, 39), nor does it explain the activity of the 2,6-dimethyl-3-nitro- and 2,6-dimethyl-3-halogen-substituted benzoic acids in the pea test reported by Veldstra & van de Westeringh (63) or their activity in the elongation of *Avena* coleoptile sections (40).

Veldstra (59) has found that the ultraviolet absorption spectra of the di-*ortho*-substituted compounds indicates steric inhibition of resonance with the carboxyl group forced into a position at right angles to the benzene ring. This finding would substantiate his hypothesis of activity dependent upon a nonpolar portion of the molecule (ring system) associated with an acidic polar group (carboxyl group) situated out of the plane of the ring as far as possible or as frequently as possible (62, 64). However, many di-*ortho*-substituted benzoic acids are inactive in the pea test and the coleoptile test (38, 59). Most of these compounds have a substituent in the *para* position and it appears that substitution at this position eliminates stimulatory activity in elongation irrespective of the spatial position of the carboxyl group or the nature of the *ortho* substituents. However, the complete lack of activity of 2,6-dimethoxybenzoic acid (38) is directly opposed to the hypothesis that di-*ortho* substitution brings about activity simply by holding the carboxyl group out of the plane of the ring. It is possible that di-*ortho* substitution of the benzoic acids forces reaction at the *meta* position when a halogen atom or nitro group is present, as suggested above for reaction at the *para* position in di-*ortho*-substituted phenoxypropionic acid.

The lack of activity of phenoxyacetic acids, phenylacetic acids and indoleacetic acids substituted in both *ortho* positions is certainly opposed to any general interpretation of effect on elongation caused by the holding of the carboxyl group out of the plane of the ring. Whereas, 2,4,6-trichlorophenoxyacetic acid is completely inactive in stimulating elongation of coleoptile tissue, 2,4,5-trichlorophenoxyacetic acid is very active.

Although the hydrophilic/lipophilic balance of the molecule may be of real import in penetration, etc., an explanation of the difference in activity of these molecules in terms of hydrophilic/lipophilic ratios and consequent differences in adsorption characteristics is quite unlikely. When such characteristics were investigated with the dropping mercury electrode of the polarograph as suggested by Veldstra (58, 62), Paleg & Muir (45) found that the suppression effects on the oxygen maximum were in no way correlated with the effects of the molecules on elongation. Small suppression effects were found for substituted benzoic acids causing elongation and great suppression effects were found for substituted phenoxyacetic acids not inducing elongation. Furthermore, within a series of related compounds an active molecule may have a suppression effect that is greater, less or the same as the suppression effect of inactive molecules.

Muir & Hansch (38) have pointed out that the benzoic acid ring is electron deficient in contrast to the electron rich rings of phenylacetic, phenoxyacetic, and indoleacetic acids. Such consideration indicates that the electron rich and the electron poor rings would vary greatly in their tendency to be held at a biological interface. The specific type of ring structure required to confer regulator activity indicates that simple physical adsorption is an inadequate explanation of the mode of action. Although cyclohexene acetic acid has been reported to have some activity in the pea test (69), no other regulator has been found which does not have an aromatic ring. The fact that compounds such as cyclohexane and decahydronaphthalene with saturated, nonaromatic rings are not active (62) is quite surprising if one assumes that the function of the ring is merely physical adsorption. A systematic attempt by Veldstra & Booij (62) to find acids with nonaromatic rings which possessed activity was unsuccessful.

If one regards the ring structure as a site for substitution at an *ortho* position, then the nonactivity of the nonaromatic compounds is readily explained since aromatic rings are unique in undergoing a substitution reaction with the displacement of a hydrogen atom. This mechanism has been discussed in detail elsewhere (38, 39) where it was shown that H^+ would be easily removed from phenoxyacetic acid by an oxidative displacement while this same mechanism would not operate in the benzoic acids since the H^+ is extremely difficult to remove from the electron-deficient ring. Thus, the activity of the benzoic acids depends upon the presence of a group of the type X^- (halogen or nitro) at the *ortho* position which can be displaced easily.

The activity noted for benzoic acids with methyl groups in both *ortho* positions does not necessarily invalidate the two-point reaction hypothesis as has been claimed by Veldstra (60). Methyl groups have been shown to be displaced under mild conditions from compounds such as 2-methyl-4-methoxy-1,4-benzoquinone (13). Also it is conceivable that the methyl groups could be oxidized to carboxyl groups and this electronegative group would undergo the displacement reaction.

Nature of the Growth Reaction

An hypothesis relating chemical constitution to growth regulator activity necessarily describes reactivity characteristics of the plant substrate. If the growth regulator induces elongation by consummation of a two-point attachment through the carboxyl group and an *ortho* position, it is very likely that the substrate reacts at a basic group and a nucleophilic group. If the substituted benzoic acids can induce elongation, the minimum distance between reactive groups on the substrate must be sufficient to allow for combination of such acids through the carboxyl group and an *ortho* position. The cysteinyl structure fits these requirements and the sulfhydryl group as the nucleophilic reactant provides an explanation for the selective inhibition of elongation by iodoacetate and other reagents reacting with sulfhydryls as well as the inhibitory effects of unsaturated lactones on elongation of plant cells (24). The selection of the sulfhydryl group as the nucleophilic reactant is indicated also by the reports of reaction between coenzyme A and indoleacetic acid as well as other auxins presumably through formation of thioethers (28, 48).

McRae & Bonner (33) have noted the fact that the inactivity of 2,4-dichlorophenoxyisobutyric acid cannot be explained on the basis of steric hindrance by the α-methyl groups preventing approximation of the carboxyl group and *ortho* position. They have thus proposed another characteristic of the substrate in the form of a barrier between the two positions. The lack of activity of the compound may, however, be due to failure of formation of the requisite amide linkage (24) as the α-methyl groups may permit salt formation between the carboxyl group and basic amino group but prevent subsequent amide formation.

The nucleophilic condensation at the *ortho* position with a sulfhydryl group of the substrate would not be expected to be reversible. Thus, compounds with auxin activity but of low V_{max}, such as 2,3,5-triiodobenzoic acid in which nucleophilic condensation occurs, should display nonreversible antagonism as has been reported (11). Similarly, the interference of aryloxy compounds with free *ortho* positions but lacking a carboxyl group would not be expected to be overcome by added auxin although McRae & Bonner (33) have reported that the inhibition of 2,4-dichloroanisole is reversible.

Inhibition of elongation resulting from the reaction of unsaturated lactones such as coumarin with sulfhydryl groups of the cysteinyl units should not be reversible and Bonner (11) has found that it is not. McRae & Bonner (33) have pointed out that the inhibition induced by maleic hydrazide is not competitive and it would not be if the molecule reacts with sulfhydryl groups. Van Overbeek (44), however, has reported that the inhibitory effects of N-2,4-dichlorophenylmaleimide and related maleimides are reversible. He found that if sections of maize coleoptiles elongating in the presence of indoleacetic acid and either *trans*-cinnamic acid or the maleimide were trans-

ferred after five hours to similar solutions in which the concentration of the indoleacetic acid were 100-fold greater, their subsequent elongation took place at the same rate as did that of sections transferred from low to high concentrations of indoleacetic acid without inhibitor. Such results would also be obtained if the elongation of the sections was determined, not by the actual number of reactive sulfhydryl sites at any one time but by the continuous production of such sites. Reversibility of sulfhydryl reaction would not be involved. Similar considerations may apply also to those instances of limited reversibility reported for such compounds as 2,4-dichloroanisole, in which the competition is for newly formed sulfhydryls and not for those having undergone nucleophilic condensation.

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THE PHYSIOLOGY OF FLOWERING[1,2]

By James L. Liverman[3,4]

Departments of Biochemistry & Nutrition and Plant Physiology & Pathology, Texas Agricultural Experiment Station, College Station, Texas

Introduction

Three years have passed since this subject was last reviewed in the *Annual Reviews of Plant Physiology* by Lang (1). Although other reviews have appeared in print since that time (2, 3), the literature has not been reviewed past December, 1951. During the interim, several important facts have been reported. Thus, new insight has been gained into the nature of the low intensity light reaction; auxin has been clearly demonstrated to participate in the flowering response of both LDP[2] and SDP[2]; a second high intensity light reaction has been shown to exist in SDP; other findings of significance have been made concerning the role of auxin in the flowering process; and further confirmation of the identity of the flowering hormone, florigen,[2] in LDP and SDP has been achieved.

The purpose of this review is to integrate these new findings and the older information into a coherent picture of the physiology of flowering, except that the new advances in vernalization will not be discussed. The discussion will revolve principally around the partial processes in the flowering response of the SDP, *Xanthium pennsylvanicum*, since much off the recent work has been done with this plant. With *Xanthium* as a point of departure, the flowering of LDP will also be discussed. Finally, an attempt will be made to bring together the views of several workers as a means of orienting future research on flowering physiology and biochemistry. It is obvious from the start that any attempt to construct a general theory of photoperiodism which will stand the test of time is doomed. Any formulation or proposal made, then, is only tentative and is intended to serve as a working hypothesis for the systematic investigation of the photoperiodic response.

In the past it has proven experimentally advantageous to divide the complex photoperiodic response into a series of sequential partial processes, so that each may be studied in more intimate detail. In this review the flowering response has been divided into partial processes which are readily recog-

[1] The survey of the literature pertaining to this review was concluded October 15, 1954.

[2] The following abbreviations will be used: LDP, long day plant(s); SDP, short day plant(s); Florigen, flowering hormone.

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nizable under natural environmental conditions, i.e., (*a*) high-intensity light process, (*b*) dark process, (*c*) second high-intensity light process and the processes (*d*) of hormone translocation, and (*e*) of bud differentiation. Each of these processes, in turn, is constituted by a series of partial reactions. It is these individual reactions, as they constitute a part of the basic process, which will concern us here. Let us first briefly consider a part of the evidence for the existence of these individual partial processes, and then proceed to discuss each in some detail in light of the additional knowledge which has come to us during the past three years.

The requirement for a period of high-intensity light with SDP can be shown with plants such as the soybean, which requires more than one inductive cycle, simply by altering the intensity of light during the photoperiod between the first and second dark periods (4). Since the intensity and duration of this photoperiod are such that an appreciable amount of photosynthesis can occur, the requirement for the period of high-intensity light appears to be a requirement for respiratory substrates. Indications are that this process is also operative in LDP.

The need for a dark period of some minimal length in SDP is shown by the fact that in 24-hour cycles SDP will not flower unless given a dark period longer than some minimum. Thus, cocklebur will remain vegetative for many months when exposed only to 16-hour days and 8-hour nights; however, exposure to a single uninterrupted night longer than 8.5 hr. results in flowering (5). This dark period must be continuous since interruption by a brief period of low-intensity light nullifies its promotive effect (5). LDP apparently do not require a dark period, although in the natural environment they are almost always exposed to one. Dark periods longer than some critical duration inhibit flowering but a period of low-intensity light during darkness reverses this inhibition and the LDP acts essentially as though it were in continuous light (6). This minimal evidence indicates that the dark period is very important in the flowering processes of both LDP and SDP and that our understanding of the inductive mechanism may depend largely on our understanding of the reactions constituting this process.

Evidence for the existence of a second high-intensity light process in the flowering of SDP has accumulated only recently. Hamner (7), Mann (8), and Snyder (9) observed in 1940 that inhibition of flowering in SDP resulted when an otherwise inductive dark period was followed either (*a*) by a flash of light followed by more darkness or (*b*) by continuous low-intensity light for some time. These results were not further considered until Lockhart (10) in Hamner's laboratory, showed that the response is a reproducible one and that for optimal flowering, the dark period must not be followed either by low-intensity light or by a flash of light followed by a period of darkness. These observations have been confirmed by Salisbury (11). Cocklebur plants will flower even in continuous darkness, showing that there is no absolute dependence on the second high-intensity light process; this second period of light does result, however, in a marked quantitative promotion of flowering.

This reaction has not been reported in LDP, although with improved experimental techniques it may be possible to demonstrate it.

At present, determination of the qualitative and quantitative aspects of flower hormone production depends on translocotion of the hormone from the leaf which perceives the day length stimulus to the responding bud where its presence and amount are bioassayed by the degree of differentiation of the terminal meristem. Thus, in one respect, the processes of translocation and differentiation are complicating reactions which are important only because they serve to assay the degree of success of the other processes under discussion. In other respects, however, they may play more than a passive role in the processes leading to induction.

The brief evidence outlined above shows the existence of several partial processes in the photoperiodic response. This information may be summarized as shown in Equation 1:

$$\text{High-Intensity Light Process} \rightarrow \text{Dark Process} \rightarrow \text{2nd High-Intensity Light Process} \rightarrow \text{Translocation and Differentiation} \qquad 1.$$

This equation represents both the sequential nature of the processes as they are thought to occur in the plant as well as the order in which they will be discussed in the following section.

PARTIAL PROCESSES OF THE PHOTOPERIODIC RESPONSE

The High-Intensity Light Process

That the need for high-intensity light in the flowering response is principally a requirement for photosynthesis, is indicated by two lines of evidence. In the first place, not only are the intensities of light involved quite high but it has been shown (12, 13, 14) that CO_2 must be present in the air surrounding the leaf undergoing induction during the time the light is given. In the second place, Liverman & Bonner (15) have shown directly with one short day-plant, that the need for a period of high-intensity light can be obviated by treating the leaves with respiratory substrates such as sugars and organic acids of the Krebs cycle.

The light energies needed for the high-intensity light process of SDP has been shown to be of the order of 1×10^6 fcm (foot candle minutes) (4, 7, 9). The energy required by LDP has been determined only for a single plant and is of the order of 10^6 fcm of light in each 24-hour cycle (16). Although it has not been shown clearly with LDP that the requirement for high-intensity light can be replaced by respiratory substrates, Klein & Leopold (17) have reported that the addition of organic acids to LDP during photoperiodic induction results in a promotion of flowering. This is a quantitative response in which the number of flowers is increased approximately 20 per cent by the addition of these substrates. In addition, it has been shown by Lang & Melchers (18) that sugar infiltration of the leaves of *Hyoscyamus niger* results in flowering under conditions where the controls remain vegetative.

In SDP, the promotive effect of this first high-intensity light process is completely understandable on the basis of a production of energy substrates. It is fairly obvious, however, that during this high-intensity light period, at least two additional things occur which apparently are inhibitory to the induction of SDP. Thus, there appears to be a net increase in the auxin level (19, 20, 21) and a shift in a pigment reaction system to a condition unfavorable for induction (22). In the case of the pigment reaction system, however, we know that high-intensity light is not necessary to induce the shift since low-intensity light is also quite effective (22). This latter reaction will be discussed in more detail in connection with the low-intensity light reaction of the dark process. The present evidence indicates that the same three reactions occur during a period of high-intensity light in LDP but, in this case, the reactions promote, do not inhibit, flowering since continuous light is better for flowering than any other regime [see references in (1)].

In addition to this promotive high-intensity light process there appears to be a promotive low-intensity light process in SDP. Thus, Harder & Gummer (23) and Lang (24) have shown that SDP which do not flower in continuous darkness can be induced to flower by interrupting this continuous darkness with 100 to 800 fcm of light given once each 24 hr. Nothing is known of the biochemical nature of this light reaction. The energies involved certainly are not sufficient to induce any appreciable amount of photosynthesis although it is possible that this amount of energy could induce the formation of adenosinetriphosphate (25). This decision must await further study of the energies of light needed to cause the fixation of inorganic phosphate into the pyrophosphate bonds of ATP. Another possible interpretation of this function of light in the flowering of SDP was given by Liverman & Bonner in 1953 (26). They suggested that this light flash reaction promotes flowering by causing the formation of an auxin receptor which combines with auxin to form the growth active auxin-receptor complex. This growth active complex, which will be discussed in connection with the low-intensity light reaction of the dark process, could be the rate limiting reaction since a small continuing supply of energy could arise from hydrolysis of storage materials. The validity of the above suggestions must await further research.

The Dark Process

Introduction.—By definition, SDP are those plants which require more than some critical amount of uninterrupted darkness in each 24-hour cycle for flowering to occur. Thus, a cocklebur plant may be kept vegetative for many months when grown on a regime of 16 hr. of light and 8 hr. of darkness daily (5). It is important, however, that exposure to a single dark period longer than 8.5 to 9 hr. results in the initiation of flower buds at the terminal meristem, whether the plant is given continuous light or continuous darkness following this inductive cycle. *Xanthium* is unique in this respect, however, in that most other SDP require two or more cycles of short days before initiation will follow under long-day conditions. This is true, for instance, of

the soybean, *Perilla*, *Kalanchoe*, and others. This uniqueness in the behavior of *Xanthium* is not a hindrance to study, but rather is a very great convenience in that experiments can be run using a single inductive cycle and thereby eliminate difficulties in interpretation which are encountered with plants which require more than one inductive cycle. With LDP it has never been possible to show a requirement for a dark period. On the contrary, all data indicate that continuous light is the most favorable condition for flowering of all LDP and dark periods are inhibitory to flowering [see references in (1)]. Thus, it appears that in the reactions which go on in the dark period are to be found the answer to the question of the biochemical differences between LDP and SDP.

What do we know about this all-important dark process and the photoperiodically important reactions which take place during it? What may we infer about the biochemical nature of this dark process in SDP and LDP from the accumulated facts? These facts show that to be inductive for a SDP, a dark period not only must exceed some minimum length, but to be maximally effective, it must not exceed some optimum length. The dark process appears to have a requirement for CO_2 and it is inhibited by low temperature. Two aspects of the dark process which make it especially adaptable to study, are that it is inhibited by applied auxin or by a flash of low-intensity light. Let us now take up each of these partial reactions of the dark process in turn and discuss it in detail.

Minimum and optimum length.—The fact that a minimum dark period is necessary for induction has already been indicated, but the biochemical reactions which determine this need are mostly unknown and one can only conjecture as to their nature. The many factors which appear to govern minimum night length will be brought out in the course of our discussion of the reactions which go on during the dark period.

The optimum length for a dark period in SDP in 24-hour cycles apparently is dependent upon at least two factors, the rate of formation and the rate of dissipation of florigen in the leaf in the dark. Facts presently available suggest that these two factors are intimately related to the age of the leaf being induced. Thus, Mann observed in 1940 (8) that the degree of differentiation of the terminal bud of a cocklebur plant as measured at an arbitrary time after induction increases linearly with the length of the inductive dark period for dark periods between 9.5 and 12 hr. Further increase in length of the inductive dark period results in a decrease in the flowering response (8). Naylor, in 1941 (27) reported that young leaves have a shorter critical night length than older leaves. These results have recently been confirmed and extended by Khudairi & Hamner (28) and by Salisbury (11) to show that it is the very young, one-half expanded leaf which is most sensitive to induction. Both younger and older leaves are much less sensitive. Although the leaf size may be used as a criterion for selection of plants, under normal greenhouse growth conditions it appears to be the physiological age which is important. Thus, if the plant is topped at the cotyledonary node and the

axilliary buds allowed to develop, at least 7 to 10 days must elapse before the new leaves become sensitive to induction even with multiple short-day cycles The total leaf area at this time measures approximately 0.22 cm^2. Even at the age when all plants had flowered, the total leaf area was only approximately 1.12 cm^2. This may be compared to the 8–25 cm^2 which is the optimum leaf size for induction of plants grown under normal greenhouse conditions (28).

Salisbury (11) has further extended these observations to show that the most rapidly expanding leaf is the most sensitive to induction. Under Salisbury's experimental conditions, the minimum and optimum night lengths for this most sensitive leaf were 8 to 9 and 13 to 14 hr. respectively. By the twentieth hour the effective hormone had decreased to approximately 45 per cent of that present at the optimum night length. In a younger leaf the minimum and optimum lengths were 11 to 12 and 13 to 14 hr. respectively, and by the fourteenth hour the hormone had been reduced to only 10 per cent of that present at the optimum length. With older leaves the minimum length had increased to 13 or more hours. The optimum length for these old leaves was not determined. It is important experimentally that the rate of dissipation of florigen is of the order of 20 times faster in the younger leaves than in the most sensitive leaf. It becomes very obvious, then, that the leaves must be selected rather critically if consistent results are to be obtained in photoperiodic studies. This work has been done with *Xanthium* but this phenomenon may well apply to other SDP and possibly to LDP as well.

What do these results mean in terms of our concept of the flowering hormone? On the basis of these data we are led to conclude either (a) that there is an outright destruction of the hormone in dark periods longer than the optimum, or (*b*) that there is at all times an equilibrium between rates of destruction and synthesis, but that flowering is reduced in nights longer than the optimum because some factor needed to drive the reaction toward synthesis becomes limiting (possibly energy yielding substrates produced in the high-intensity light reaction, etc.). Other factors also appear to be involved and these will be discussed in more detail below.

Role of dark fixation of CO_2.—It was suggested some time ago by Spear (29), working in Thimann's laboratory, that dark fixation of CO_2 is an important aspect of the inductive process in SDP. This and subsequent reports (30 to 33) have shown that there is a close correlation between the photoperiodic response of *Kalanchoe* and its CO_2 metabolism. Thus, short-day treatment causes a large increase in dark fixation of CO_2; the fixation process can be induced as can flowering; a flash of light which prevents flowering also prevents development of the CO_2 fixation pattern; the time of maximum CO_2 fixation corresponds to the critical night length; and fixation stops after about 16 hr., the optimum night length for floral induction in this plant (34). Another important correlation is also found between CO_2 metabolism and flowering, i.e., exposure of the plant to high temperature during the dark period causes an evolution of CO_2, as does a light flash. An additional amount

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of CO_2 is evolved upon further exposure to light (30). This evidence indicates the existence of a photolabile and thermolabile fraction in the products of dark CO_2 fixation. It is interesting that the exposure of *Kalanchoe* (or of *Xanthium*; see later) to high temperatures (30° to 35°C.) during the dark period brings about a decreased flowering response (30, 35). This suggests that the product of dark fixation of CO_2 may be causally related to flowering and that exclusion of CO_2 during a dark period should inhibit induction.

Spear (36) was not able to secure a clear-cut inhibition of flowering by the exclusion of CO_2, possibly because the marked CO_2 metabolism of *Kalanchoe* made its exclusion impossible. Langston & Leopold (37) however, have been able to demonstrate by the use of radioactive $C^{14}O_2$ that exclusion of CO_2 from the atmosphere during the dark period causes an 80 to 90 per cent inhibition of flowering in *Xanthium* and Biloxi soybean. This inhibition is greatest when the night length is very near the critical. The absorption of CO_2 is linear for the first 12 hr., and then levels off with approximately 80 per cent of the total being taken up during the first 8.5 hr. A flash of light during the dark period causes an evolution of CO_2 in the SDP, but with barley, a LDP, flowering is relatively independent of the presence of CO_2 during the dark and a flash of light has no apparent effect on dark CO_2 fixation.

It is evident from the above data that CO_2 fixation does play some important, but unknown, function in the dark processes, particularly of SDP. Chromatography of extracts of the leaves of *Xanthium*, soybean, and barley which have fixed $C^{14}O_2$ in the dark and light, shows that the radioactive carbon is distributed in a wide variety of compounds, particularly in the Krebs cycle acids, and in phosphorylated compounds (38).

Norris & Calvin (39) have compared the early products of photosynthesis in leaves of *Kalanchoe* grown in short or long-day. These results show only that quantitative difference exist between the compounds found in the leaves of the plants grown in long-day and short-day, principally in the concentrations of phosphoglyceric acid and of the amino acids, alanine, serine, and glycine. The only working hypothesis yet suggested to account for the results with CO_2 has come from Spear, Thimann & Gregory (30, 31, 32). They suggest that the high-intensity light process is concerned with formation of a CO_2 acceptor which combines with CO_2 during the dark period. In the dark, an enzyme which catalyzes CO_2 fixation is formed. Cessation of CO_2 fixation is ascribed either to exhaustion of the supply of acceptor or to translocation out of the leaves either of the acceptor or the enzyme system (31). Both the product of fixation and the enzyme are destroyed by light but the enzyme subsequently becomes photostable.

We are still at an early stage in studies on the role of dark fixation of CO_2 as related to the inductive process. In any case, this aspect of floral induction offers us a new tool for studying the reactions which go on during the dark period. This tool does have very definite limitations, however, and it will be necessary to use more than the usual amount of discretion in interpreting

the results, particularly those concerned with the products formed by $C^{14}O_2$ fixation. Simply because a compound formed in the leaves is transported to the bud does not mean that it has anything specific to do with flowering since florigen appears to travel rather passively with the photosynthate stream (1, 40). Thus, it will be necessary to determine whether the compounds being followed show any correlation in their time of translocation with movement of florigen from the leaf. Only those compounds which show such a correlation will be of interest for the flowering response.

Temperature and the dark process.—The effect of temperature on the dark process in SDP will not be considered here since it has been reviewed in detail by Melchers & Lang (41). In general, however, very low (1 to 4°C.) or very high (30 to 38°C.) temperatures are inhibitory to flowering but intermediate ranges are quite effective in induction. The optimal temperature with SDP, although varying with species, is generally in the region of 20°C. to 25°C. The time in the dark period during which the plant is given high or low temperature apparently has little significance (11). Very little effect of temperature on critical day length is observed except with a minimum number of photoinductive cycles (42).

High and low temperatures have a more pronounced effect on the flowering of LDP. Lang & Melchers (18) some years ago, found that *Hyoscyamus* initiates flowers with shorter photoperiods when grown at low temperatures than when grown at high temperatures. Thus, the critical day length decreases from 11.5 to 8.5 hr. as the temperature decreases from 28°C. to 15°C. Liverman & Lang (43) observed that *Silene* has a shorter critical day length at 17°C. and at 30°C. than at either 14°C. or 23°C. These results serve to point out that in experiments on the effect of temperature on the flowering response, it is necessary to use a number of different temperatures in combination with a number of day lengths to obtain results which are meaningful. All results presently available suggest that temperature simply accelerates or retards specific reactions in the inductive process and that its chief value lies in using it as a tool to study these specific processes.

Applied auxin and the dark process.—The inhibition of flowering by auxin application was first demonstrated by Dostál & Hosek (44) with a day neutral plant in 1937 and in the following year Hamner & Bonner (5) extended this finding to SDP. Čaĭlahĭan (45), Cholodny (46), and Galston (47) considered that flowering is controlled in some manner by auxin, but not until Thurlow's (48) experiments in 1947 was it clearly shown that flowering in a SDP is inhibited by auxin applied during an otherwise inductive dark period. This observation has since been confirmed by many different workers in widely scattered laboratories (10, 11, 30, 40, 49 to 58). These results show that the curve obtained by plotting the per cent inhibition of flowering as a function of auxin concentration represents a saturation phenomenon (59, 60) typical of the action of auxin in growth of the *Avena* coleoptile (61) and in other growth responses. It is interesting to note in this respect that the low-intensity light reaction shows a similar saturation effect (62). Bonner &

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Thurlow (49) found the inhibition to be a true auxin response in that antiauxins are able to reverse the inhibition. This result has been confirmed by Li (53) in the same laboratory.

Prior to 1952 (2), it was assumed that auxin inhibits flowering only when applied during photoinduction since application four (53), six (47), or nine days (57) after induction had no inhibitory effect on flowering of the SDP under study. In 1952, however, Liverman (40, 59) reported that auxin still inhibits flowering in *Xanthium* if applied at the end of the dark period or for some 7 to 15 hr. into the light period following this dark period. Salisbury (11, 63) has confirmed these results in detail. The inability of some workers (10, 56) to confirm these observations apparently resides in slight differences in technique. Auxin appears to be absorbed into the leaf only so long as the leaf is wetted by the auxin solution (11), therefore, the solution must not be allowed to dry rapidly or absorption of the auxin is hindered. In the experiments of Liverman (40) and of Salisbury (11) also, auxin solutions neutralized with KOH were used. The differences observed by the two groups of workers do not appear to be an effect of pH (11) but they may be a function of the potassium ion which, as shown by Cooil (64), stimulates auxin action.

Using the defoliation technique of Khudairi & Hamner (28) to follow the rate of translocation of florigen out of the leaf, Salisbury (11) has shown that auxin becomes ineffective in preventing flowering the moment all florigen has moved out of the leaf. Auxin applied to the tip also causes inhibition only as long as florigen is in the leaf. It appears then, that auxin does not inhibit flowering by preventing translocation of florigen out of the leaf but that its actions is more specific—possibly bringing about a destruction or inactivation of florigen as suggested by Liverman (40, 59).

The experiments cited above suggest that one of the factors controlling the critical night length in *Xanthium* is the auxin level in the leaves. This implies that auxin inhibition is a function of night length, i.e., a given auxin concentration causes less inhibition with a longer night length. Experiments of the author (59) appeared to support this view, but Salisbury (11), in extending these observations, has obtained results which indicate that the effect of auxin is principally upon the degree of induction and not upon the critical night length. He (11) has further observed that auxin has its greatest effect when applied 2 to 4 hr. after the beginning of the dark period. The effect of auxin applied after this time gradually lessens as the night proceeds. These results imply that auxin may have its effect both by interfering with synthesis and by inactivation of the flowering hormone or its precursor, once formed.

At present all that we know about the mechanism of the inactivation of the flowering hormone by auxin is that this is a typical auxin response, a saturation process, and one reversed by antiauxins. A final understanding of this role of auxin may become evident only after the mechanism of auxin action is understood and this knowledge may well come from studies of

simpler systems such as the *Avena* coleoptile or pea stem sections wherein the variables can be more precisely controlled. Apparently, auxin is intimately linked with the utilization of adenosine triphosphate in growth processes in plants (65) so the suggestion by Becker (66) and subsequently by Bonner (67) that auxin may inhibit flowering by redirecting the utilization of adenosinetriphosphate toward other energy-using processes seems a reasonable one. This suggestion has, by no means, been proven but the fact that dinitrophenol, a known uncoupler of oxidative phosphorylation, causes an inhibition of flowering is suggestive of this possibility (53).

The preceding discussion has shown clearly that auxin inhibits flowering of SDP when applied to the leaves prior to complete translocation of the hormone out of the leaf. An interesting aspect of the problem is that auxin applied after translocation is complete actually promotes flowering (11). That this effect operates directly upon the bud has been shown by applying auxin to the tips of plants which have been induced and then defoliated. Plants whose tips receive auxin develop flower buds at a faster rate than untreated controls.

Additional evidence bearing on this point has been brought out by experiments designed to show that a plant, to be induced, must possess an actively growing bud, i.e., an active bud as it is known in the literature. Lona, in 1949 (68), reported that leaves detached from the plant but attached to a stem segment from which the buds have been removed, can be induced with short-day treatment. This leaf causes flowering when subsequently grafted to a vegetative plant. In attempts to repeat Lona's experiments, Carr (69, 70) observed that flowering resulted from grafting this leaf to vegetative plants only if adventitious buds have developed in the axils of the leaves of the debudded segment during inductive treatment. Carr concluded, as had Gregory earlier (71), that some florigen precursor formed in the leaves in the dark is unstable in leaves in the light and is dissipated in the plant until "fixed" by the growing point. A possible objection to Carr's results is that he failed to use leaves induced prior to removal from plants as controls for his experimental treatments. Leaves induced on the plant by short-day tretment are known to cause flowering when grafted immediately to vegetative plants (3, 72, 73). There is no assurance, then, that induced leaves under Carr's experimental conditions would cause flowering when regrafted to vegetative plants. Lang (24) has mentioned preliminary experiments which indicate that detached leaves may be induced. The issue, then, of whether detached leaves can be induced and regrafted to a vegetative plant to cause flowering is still not clear. Experiments described below indicate that such an experiment would be successful under optimum conditions, particularly of time.

Lincoln (74) independently confirmed Carr's observation that an active bud is necessary for induction and, more recently (75), he has shown that very young leaves can replace the requirement for buds. Salisbury (11) has also confirmed the observations of Lincoln and has found, in addition, that

auxin in lanolin paste can substitute for the young leaves. The technique used by Salisbury was to remove the growing tip (leaving one axilliary bud) the day before inductive treatment and to apply auxin to the stump before and at various times after induction. In one experiment, 90 per cent of the plants which had been treated with auxin 2 or 3 days after the inductive cycle, flowered, whereas control plants without auxin flowered in only 45 per cent of the cases. Auxin applied five days after inductive treatment was without effect. Although these experiments of Lincoln and Salisbury are not as unequivocal as would be experiments with induction by detached leaves, they do indicate that auxin is involved in the "fixation" or "stabilization" of florigen. They further indicate that the half-life of florigen in *Xanthium* is of the order of 2.5 to 3.0 days in the absence of a growing bud. One may speculate as to whether the lack of systemic flowering in some plant species is caused by a slow rate of translocation of florigen from the leaves to a growing bud, which results in dissipation of florigen before it is "fixed." These experiments suggest that auxin application after induction might overcome this difficulty, although there are alternate explanations for the localization of flowering in some plants. Other aspects of auxin metabolism in the flowering response of SDP will be brought out in connection with the discussion of the low-intensity light reaction of the dark process.

The relation between auxin and floral induction in LDP has not been investigated as extensively as with SDP. In spite of this neglect, we now know that auxin promotes flowering in some LDP and actually causes induction under conditions in which the controls remain vegetative. The first clear demonstration of promotion of flowering in LDP by auxin application to the leaves during inductive treatment was reported by Leopold & Thimann in 1949 (52) and confirmed by Li in 1950 (53). Reports of Hussey & Gregory (76) recently confirmed these results for barley, but they report that Petkus rye is unaffected by auxin. Both groups showed that low auxin concentrations increase the number of flowers whereas high concentrations inhibit flowering. Inhibition or delay of flowering also has been observed by von Denffer & Grundler (58) who used relatively high concentrations of auxin. Subsequently, Claes (77) used a number of different threshold treatments in attempts to show an effect of auxin on the flowering of *Hyoscyamus*. She was able to achieve only a slight promotion of flowering with auxin as measured by a reduction in leaf number. It remained for Liverman & Lang (78) to show conclusively for the first time that the LDP, *Hyoscyamus* and *Silene*, can be caused to flower by auxin application under conditions in which the controls remain vegetative. In these experiments *Hyoscyamus* plants were given an 8-hour day in high-intensity light supplemented by 16 hr. of light of very low intensity. In light of such a low intensity that the nonauxin-treated controls remain vegetative, every plant is caused to flower by treatment with 3 to 10 mg./l. of auxin. We may assume, then, that this experiment demonstrates a synergistic effect between light and auxin, i.e., there is an intensity of light and a concentration of auxin such that either alone is

ineffective, but such that both together cause a marked inductive effect. There is a great need for additional studies on the role of auxin in the floral induction of other LDP, particularly to see in how far these results with *Hyoscyamus* and *Silene* may be extended to other LDP.

Recent evidence indicates that auxin is not only of interest in connection with floral induction, but that it may play an important role in the process of elongation of the central axis of LDP. Most LDP remain in a basal rosette under short-day conditions but begin to elongate rapidly when placed under long-day conditions. This elongation is usually correlated with the onset of floral induction. It was shown some years ago, however, by Murneek that this is nothing more than a coincidence since with *Rudbeckia* the processes of elongation and initiation can be separated (79, 80). Murneek observed that one can obtain flowers in a rosette or elongation without flower formation depending upon the temperature conditions. Murneek suggested that this response might be a reflection of the differential action of photoperiod on growth substances (80). Application of auxin to *S. armeria* [LDP (40)] under long-day conditions causes a marked acceleration of the rate of elongation of the central axis. A report by Konishi (81) indicates again that elongation is an auxin phenomenon. He observed that the ability of stem and leaf tissue of *S. armeria* to convert tryptophan to auxin as determined by the *Avena* test is much greater in bolted plants than in those still in a rosette. In addition, the activity of indoleacetic acid (IAA) oxidase is much reduced in the bolting plants as compared to those in a rosette. This is true both as determined with the purified enzyme and as determined by diffusion of IAA through stem segments. Unfortunately, Konishi has not reported a quantitative basis for comparison of the various activities, i.e., he has not compared the activities on a protein, dry or fresh weight, or other basis. His results do indicate that the bolting plants not only produce more auxin but destroy less of it as compared to the vegetative plants in a rosette.

To summarize, then, it appears from data presently available to the author, that the auxin level goes down during the dark period in both LDP and SDP. In SDP the dark period allows the concentration of auxin to fall to a level where florigen synthesis can occur. For LDP, on the other hand, the data suggests that their auxin level may be too low for flowering in short-day conditions and that by making up this deficit artifically, flowering may ensue.

No direct correlation has ever been shown to exist between the kinetics of the photoperiodic response and the fluctuations of the native auxin level in the plant [see references in (2)]. Recently, Khudairi & Bonde (82) reported the existence of a growth inhibitor in the leaves of *Xanthium* which showed a reasonable correlation with the kinetics of the flowering response. Subsequent work in the same laboratory with a better assay procedure, however, has failed to confirm this correlation (83). It is possible, of course, that the active form of auxin is not free auxin but is auxin attached to some receptive entity in the plant cell. This would make it unlikely that any ordinary chemical

analysis of the leaf would measure the actual effective auxin. It would appear more promising to look for an independent measure of its activity such as rate of water uptake, leaf movements, etc., until such time as a procedure for isolation and quantitative determination of the growth active auxin-receptor complex has been developed.

The low intensity light reaction of the dark process.—Further important knowledge of the dark process has come from studies aimed at understanding the nature of the chemical reactions which apparently are reversed by a light interruption of the dark period. The now classical observations of Katunski [LDP (6)] and Hamner & Bonner [SDP (5)] showed for the first time that a flash of light given in the middle of a long dark period promotes the flowering of LDP and inhibits the flowering of SDP. Since that time, extensive studies of this reaction have shown its photochemical nature, its action spectrum, something of its biochemical nature, and have indicated its controlling influence on a wide variety of morphological responses. The discussion in this section will concern our knowledge of this reaction and its significance in the flowering response.

Prior to 1952, a series of studies established that approximately only 10 to 100 fcm of light at the wave length of maximum sensitivity are required for consummation of the low-intensity light reaction (84 to 89). The temperature independence of this reaction was established (90 to 94) and an action spectrum was known for the flowering of SDP (87) and LDP (84, 89), as well as for promotion of leaf expansion (88) and inhibition of internode elongation in seedlings (85, 88, 93 to 98). The action spectra for these responses are similar with maximum effectiveness in the red near 6500 Å. The curve for spectral efficiency decreases to a minimum near 4600 Å.

Since early 1952 significant progress on the nature of this low-intensity light reaction has come to us from the now classical experiments on lettuce seed germination by the Beltsville group (99). Flint & McAlister, in 1935 (100), reported that lettuce seed germination is maximally promoted by light in the red and is maximally inhibited by irradiation in the 7100 to 7500 Å region of the far red. The Beltsville group set about to determine the similarity between the action spectrum for this response and for other light responses already known. The spectrum for promotion of germination was obtained by imbibing the seed in the dark followed by exposure of the seed to light of different regions of the visible spectrum. The spectrum for inhibition was obtained by taking seed previously fully promoted by red light and exposing them to different portions of the spectrum. The results confirm the observations of Flint & McAlister (100) and show that the action spectrum is identical to that for other morphological responses (i.e., maximum effect near 6500 Å) except for the additional response of the lettuce seed to exposure in the far red (maximum activity at 7350 Å). Borthwick and his coworkers immediately recognized that their earlier spectrum determinations had never been conducted in a manner which would detect a response to the far red portion of the spectrum, provided that this response were opposite to

that elicited by red light. It is interesting to learn in this regard that, although reports on the effect of far-red in controlling growth responses appeared prior to 1952 (88, 93, 98), the existence of a reversible reaction apparently was not suspected. The Beltsville group found that the far-red portion of the spectrum reverses the inhibition of flowering of cocklebur produced by red light and would, in addition, shorten the critical night length by some 2 hr. if given during the last 30 min. of the light period (22). Subsequent reports have amply confirmed these observations of the Beltsville group and have shown, in addition, that an action spectrum similar to that for lettuce seed germination (99) and the flowering of *Xanthium* (22) holds for the germination of *Lepidium* seed (101), for leaf expansion and hypocotyl growth of dark grown beans (102), and for coloration of tomato cuticle (103).

Possibly the two most important and useful facts to come from these studies are (*a*) that the germination response depends solely on the last of a sequence of exposures to red and far-red irradiation, i.e., if red light is given last, germination is promoted, if far-red light is given last. germination is inhibited; and (*b*) that the germination response following the first and the final exposure either to red or far-red light is essentially equal. This reversibility appears to be a general property of all low-intensity, light-mediated responses reported to date (22, 25, 99, 105), provided only that the time between exposure to red and far-red is kept to a minimum. More detailed experiments on the action spectrum have shown (105) that seed brought to full germination can be inhibited or, conversely, those fully inhibited can be promoted by exposure to radiation in the 4000 Å to 5000 Å and the 7000 Å regions of the spectrum except that about one hundredfold more energy is required at 4000 Å to 5000 Å than at 7000 Å. Prolonged exposure to either of these spectral regions alone brings the seed to an intermediate equilibrium value for germination.

The results outlined above indicate either that two separate pigments or two forms of the same pigment are involved in light perception, one absorbing maximally near 6500 Å, the other maximally near 7350 Å, with both having overlapping regions near 4000 Å to 5000 Å and 7000 Å. It appears likely, although it is by no means proven, that the system consists of two forms of the same pigment. The facts which suggest this conclusion are (*a*) germination response following the first and final exposures either to red or far-red light is essentially equal; (*b*) high temperature causes a reversal of red light promotion, possibly by causing a decay of the far-red absorbing pigment to the red absorbing form; and (*c*) there is a change of sensitivity to light-induced germination as a function of the time of imbibition before exposure to radiation, i.e., as germination response to a given red exposure increases there is a concommitant decrease in the germination response to a given far-red exposure and visa versa. On the basis of this shift in sensitivity with time of imbibition, Borthwick *et al.* (22, 99) concluded that a reactant other than pigment is involved. Subsequent experiments conducted in the Beltsville laboratory (101, 102, 103, 105) show that the response under study

may escape from photochemical control if the time between exposure to red and far-red is sufficiently long. Low temperature slows the rate of this escape from photochemical control indicating a linking of the photochemical reaction to a thermal reaction. These results led to the postulation of still a third reactant (101, 106, 107). Reasoning along different lines from work with the *Avena* coleoptile section, Liverman & Bonner came to much the same conclusion [(26) see also p. 193]. The reactions postulated by the Beltsville group are indicated as follows:

$$\text{Pigment } (P) + \text{Reactant } (RA) \underset{7350\ \text{Å}}{\overset{6500\ \text{Å}}{\rightleftharpoons}} PR + A \qquad 2.$$

$$PR + \text{Molecule } (M) \xrightarrow[\text{Dependent}]{\text{Temperature}} PRM \qquad 3.$$

In this formulation, *PRM* would be the form of the pigment important in the control of seed germination, of flowering, etc.

In how far do these relations hold for the flowering response? Borthwick and his co-workers have shown that the inhibitory effect of a flash of red light in *Xanthium* can be immediately reversed by a subsequent flash of far-red (22). Downs (102), in extending this work, has shown clearly that the primary photoreaction of flowering is coupled to a temperature-dependent reaction. Thus, the ability of far-red to reverse red light-produced inhibition of flowering in *Xanthium* depends on the length of the intervening dark period. At 20°C. there is a direct proportionality between elasped time and the degree of reversibility. After approximately 35 min. far-red becomes completely ineffective. One-half reversal is still possible after 60 min. when the plants are kept at 5°C. following irradiation. Extrapolation of the curve produced by the experimental points shows that the ability of far-red to reverse the red inhibition should be lost after about 2 hr. at 5°C. (102). Apparently, the same reversible reaction controls flowering in LDP (108).

One may inquire as to the most appropriate way to carry out this low-intensity light reaction in the flowering response. It has been amply demonstrated that very low energies of light are effective in consummating the low-intensity light reaction in both LDP (84, 89) and SDP (87). Furthermore, a light flash is just as effective as continuous light in promotion or inhibition of flowering when given at the appropriate time during the dark period. The choice of technique, then rests on the particular experiment to be conducted. Kinetic studies in the past show that the most effective time for exposure to the flash is in the middle of the dark period (84 to 89, 109). Salisbury has found recently, however, that maximum inhibition is achieved in *Xanthium* between the fourth and ninth hour of darkness for dark periods varying in length from 10 to 20 hr. (11). With a 10-hour dark period, a 12-second flash is equally effective from the fourth to the eighth hour, with 12 hr. of darkness, from the sixth to the eighth hour, and in 16- and 20-hour dark periods from the seventh to the ninth hour. Apparently, the period

between 6 and 8 hr. of darkness is very critical in the dark process. It is interesting in this regard to find that Lockhart & Hamner (56) have observed that the degree of inhibition by a flash of light given after a 10-hour dark period is directly related to the length of dark period following this flash.

Before light can be effective in a reaction system such as that described above, it must be absorbed by some pigment which can utilize the light energy to do chemical work. Prior to the discovery in 1952 (99) that light controls many important morphological responses through a reversible photoreaction, it was generally assumed that the photoreceptor was an open chain tetrapyrrole such as phycocyanin (85). The discovery of the reversible nature of the photoreaction, however, called attention to the possibility that the photoreceptor might actually be a reversibly reduced or otherwise altered porphyrin. Todd & Galston (110) following this lead found a pigment with some of the properties expected of the photoreceptor in numerous non-chlorophyllous photosensitive tissues. The extracted pigment, which resembles methyl pyropheophorbide-*a*, is present in smaller amounts in seeds irradiated with red light immediately before extraction than in those treated with darkness or far-red light. In addition, they found a reasonable correlation between content of this pigment in the seed and the degree of germination in response to red light. There are, on the other hand, some indications that this particular pigment is not the photoreceptor. The extracted pigment shows a maximum in the red at 6670 Å whereas the action spectrum shows a maximum at 6500 Å. The pigment has no peak in the far-red, and the absorption in the blue is much too great as compared to that in the red. These latter discrepancies can be reasonably rationalized as Todd & Galston have done, but as final proof that this is the photorecetpor, it would be desirable to show that irradiation with light at 6600 Å leads to a disappearance of the red peak with a concommitant appearance of the far-red peak at 7350 Å. Although it will probably be some time before this reaction is carried out *in vitro*, it should be possible to demonstrate it *in vivo* by a suitable technique. Studies of such an *in vivo* system would go far toward helping us to understand the chemical nature of the initial stages of this all-important photochemical reaction.

Even though we do not have in our hands the valuable tool described above for study of the initial photoreaction of the low-intensity light reaction of flowering, we have learned a great deal about the biochemistry of the process. In this respect, we are far better off now than we were three years ago for, during the intervening time, it has been shown that many of the light-growth reactions are controlled by the same or quite similar reaction mechanisms. The knowledge that a similar basic mechanism controls a wide variety of responses, allows us to utilize fully the information obtained from studies of any of them in understanding the flowering response.

What do we know of the biochemical nature of the light flash reaction? In 1949, Bonner (111) found that inhibition of flowering in cocklebur by continous low-intensity light can be reversed by application of an antiauxin, triiodobenzoic acid. Liverman (54) subsequently reported that the light

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flash inhibition also is reversed by the application of auxin antagonists. This gives evidence for the biochemical similarity of the two types of light action and links the light flash reaction with the plant's own auxin metabolism. After the red-far-red reversibility experiments were reported by Borthwick *et al.* in September, 1952 (22, 99), Liverman & Bonner (26) began experiments aimed directly at ascertaining the role of auxin in the response. The *Avena* coleoptile was chosen as an experimental object for two reasons, (*a*) coleoptile growth rate is increased by red light of approximately the same wave length as that which is functional in the photoperiodic response (95), and (*b*) growth is almost completely dependent upon added auxin. Furthermore, we know a great deal more about the auxin-growth reaction in *Avena* than in any other single tissue.

Growth of the *Avena* coleoptile may be described in terms of classical enzyme kinetics as shown in Equation 4 (61),

$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} ES \xrightarrow{k} \text{Growth} \qquad 4.$$

wherein, E represents the auxin receptive moiety within the plant, S, the auxin molecule, and ES, the growth active auxin-receptor complex. This system is further described by the parameters K_s and k which are respectively the dissociation constant of ES, and the rate constant which relates ES to final growth rate. In systems such as the *Avena* where neither the total concentration of E nor the absolute value of k is readily determinable, their product may be replaced by the parameter *V-max*, the growth rate under nonlimiting S concentrations wherein all E is converted to ES. That such a system accurately describes growth in the oat coleoptile has been amply confirmed (112 to 115). Conceivably, light could affect this system in several ways. Thus, it might affect the formation or inactivation of E, of S, or ES, or it might affect the combination of E with S or it might only influence the constant, k, which relates the concentration of ES to overall growth rate. By suitable experiments it was shown that red light promotes elongation of the coleoptile whether or not auxin is present during the exposure (26). On the other hand, far-red reverses the red promotion only if auxin (S) is present during the exposure to far-red. This reduced growth rate following far-red exposure is not caused by destruction of auxin (S) since a subsequent exposure to red fully restores the system to its former activity. These results suggest that red light causes the activation of an auxin receptor. This receptor per se is not susceptible to far-red inactivation, but the receptor in combination with auxin, i.e., the auxin-receptor complex, is subject to inactivation. These relations may be summarized in the following equations:

$$E_{\text{inactive}} \xrightarrow[\text{(dark slowly)}]{\text{Red}} E \qquad 5.$$

$$E + S \rightleftarrows ES \qquad 6.$$

$$ES \xrightarrow[\text{(dark slowly)}]{\text{Far-Red}} E'_{\text{inactive}} + S' \qquad 7.$$

Since red light can reverse the far-red inhibition, it appears that E_{inactive} and E'_{inactive} are interchangeable, if not identical, and both may be termed E precursor or E_p. If this is assumed to be true, reactions 5 through 7 may be simplified and combined in cyclic form as shown in Figure 1. This cyclic formulation will be referred to as the photocycle in future discussions.

The red light-induced increase of *Avena* coleoptile growth rate (26) is to be contrasted with the red light inhibition of growth of oat, pea, bean and barley internode growth (85, 88, 93 to 98, 116). Galston has shown that this

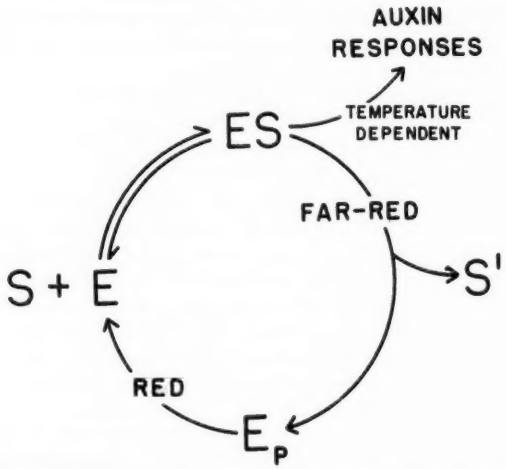

FIG. 1. The morphogenetic photocycle. Auxin (S) combines with auxin receptor (E) to form the growth active auxin-receptor complex (ES). This complex may lead to growth responses or may be decomposed by far-red light to form auxin-nonreceptive entity E_p plus some compound (S'). The auxin-nonreceptive entity E_p is again activated by red light to form (E).

light-induced growth reduction in pea is the result of a decrease in available auxin, since the inhibition in light-treated sections is reversed by increasing the auxin concentration (117). The reduced growth may be a reflection of an increased IAA destruction by red light-promoted IAA oxidase activity (117) or a decreased uptake of auxin by the tissue, or both although the decreased uptake is somewhat ambiguous (117, 118). It is interesting that the effects of light on auxin metabolism in the pea internode are not immediately evident but have a 6 to 18 hr. lag period indicating a light-induced alteration of one or possibly more enzyme systems (117).

The hypothesis that red light-induced growth inhibition is due to increased IAA oxidase activity is further supported by experiments of Miller (119) and others (120, 121) which show that the cobaltous ion promotes

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elongation of etiolated pea stem sections. Since Galston & Seigel have shown (120) that IAA oxidase activity is positively correlated with peroxide production, cobalt, which depresses peroxigenesis in pea stem tissue (120), in effect, causes a decreased IAA oxidase activity. This decreased activity is reflected in a lower rate of auxin destruction and an increased growth rate. If, as Galston suggests (120), the effect of light on growth is through its effect on peroxide production, cobalt should reverse, at least partially, the light-induced inhibition of pea stem segment growth. These data are not yet available. It is interesting in this regard, to note that cobalt does not appear to increase the growth rate of the *Avena* coleoptile (122, 123) nor of green pea stem tissue (119). In both of these tissues, *in vivo* IAA oxidase activity is low and red light promotes, rather than inhibits, elongation.

Further insight into the light-growth mechanism is found in the light-induced unbending of the plumular hook of the red kidney bean (102, 124 to 127). The unbending is due, anatomically speaking, to a faster elongation of cells on the inside of the hook than on the outside (124). Physiologically, then, light selectively either promotes growth of the cells of the inner portion of the hook, or conversely inhibits the growth of the cells on the outer portions. If light, as suggested by Galston (120), controls morphogenic responses by producing peroxides which couple with IAA oxidase to destroy auxin, cobalt should antagonize the unbending and should not cause an opening of the hook in the dark as it actually does (126, 127). A further apparent paradox is that high auxin levels inhibit the unbending, whereas low auxin concentrations promote the response (124). One additional fact observed by Miller is that light and cobalt promote elongation of bean stem segments cut from above the hook but not those from below the hook (127). These results imply that the first alternative given above is correct, and that the light reaction under consideration here is not the one which produces peroxides and thus, apparently, is not the same reaction studied by Galston in the pea stem. It is more suggestive of the photocycle reaction of Liverman & Bonner (26) in which red light causes a promotion of growth. All of the above results are understandable with the help of the latter concept. Thus, light promotes the unbending reaction by causing the formation of auxin receptor, E, which causes a shift in the equilibrium relating E, S, and ES toward formation of more auxin-receptor complex, ES. This complex in its usual role promotes growth and unbending of the hook. Under the photocyclic concept, cobalt promotes unbending by sequestering peroxide, thus preventing the action of IAA oxidase. This has the net effect of increasing the auxin, S, concentration causing a shift toward formation of more ES which promotes unbending. Although IAA oxidase has not been demonstrated in the plumular hook of bean, Galston (128) has recently shown that the hook in pea does have a low oxidase activity, therefore, it may also occur in bean. The promotion of unbending by low and inhibition by high concentrations of auxin (S) are understandable with the help of the two point attachment concept of Muir, Hansch & Gallup (129) and of Foster *et al.* (61).

The promotion of expansion of etiolated leaves by light (88, 104, 116, 122) and by cobalt (126, 127) are understandable under the same conditions as unbending of the plumular hook in the red kidney bean. Thus, ES is rate limiting to expansion and exposure to light or increase in S either by the addition of cobalt or of S, causes an increase in expansion. Although it has never been possible to consistently demonstrate promoted leaf expansion by auxin, occasional stimulation has been observed with 10^{-8} to 10^{-9} M IAA independent of light effects (123). Since young leaves are known to contain auxin (130), it might be possible to show a promotive effect by auxin only under special conditions. Cobalt and light are essentially additive in their effects on leaf expansion and Miller has suggested that this may be an effect upon the same limiting condition, but by different pathways (127). This suggestion may be understood under the photocyclic concept outlined above, i.e., light converts E_p to E and cobalt has its effect by preventing peroxigensis which, in effect, increases S. An increase in either E or S results in a shift of the equilibrium toward formation of more ES and, consequently in more cell enlargement (127) presumably by water uptake. It seems quite unlikely that auxin-light mediated responses are the only ones involved in these various morphological responses, but certainly, there is little doubt that they are important, possibly even the controlling ones.

It is of interest that application of the cobaltous ion (11, 40) to *Xanthium* during induction causes a reduction of flowering. Other peroxidase substrates, such as catechol, pyrogallol, etc., also cause inhibition of flowering (40). Thus, although it has been difficult to follow the auxin fluctuations in the leaves, these substances which have a sparing action on the oxidation of IAA (131) do cause inhibition as does auxin itself. Most of the previous discussion has concerned the effects of light and auxin and other factors in the flowering of SDP. Although few, if any, of the above described experiments have been carried out with LDP, one is led to suspect, on the basis of present evidence, that cobalt and the phenolic substances would promote flowering of LDP as does auxin and low-intensity light interruption of the dark period.

The data presented above suggest the active participation of at least two red—far-red controlled reactions in the regulation of morphological responses in higher plants. One of these reactions is promotive, the other inhibitory to growth, i.e., one is thought to cause the activation of an auxin receptor, the other to cause the activation of IAA oxidase. It appears that both of these reactions take place in nearly all plant tissues, but that in some cases the reaction involving activation of auxin receptor is difficult to demonstrate because of masking by the very active oxidase reaction. This suggestion is supported by recent data of Thomson (132, 133, 134). Thus, she has shown that growth of the second internode of oats and the second, third, and fourth internodes of pea are accelerated by exposure to light in an early stage of growth, whereas exposure at a later stage results in an inhibition (134). This observation suggests that as tissue gets older, it either (*a*) becomes less sensitive to the red light promotive reaction; (*b*) more sensitive

to the oxidase reaction; or (*c*) that sensitivity to reaction (*a*) remains constant but its presence is masked by the predominance of the inhibitory one. Galston has shown that IAA oxidase activity increases with aging of the tissue (128). This information does not allow a choice between (*b*) and (*c*) above, but it does point out that a thorough knowledge of IAA oxidase and its physiological role is important to our understanding of the various photomorphogenic effects of light.

Other information bearing on our understanding of the low-intensity light reaction of the dark process has been presented in experiments by Leopold & Guernsey (135) which show that respiration of various tissues of photoperiodically sensitive plants is affected by a reversible photoreaction controlled by red and far-red irradiation. Flowering of *Xanthium* and of soybean (5, 7), and growth of the oat mesocotyl (94) and pea stem sections (117) are all inhibited by exposure to red light. Red light given to these tissues causes a decrease in repiration (135). On the other hand, lettuce seed germination (99, 100, 105) and flowering of barley (84) are promoted by red light and exposure of these tissues to red light results in an increased respiration. These red light-mediated responses are all reversed by exposure to far-red. These results, while apparently correlated with photoperiodic phenomena, are not extensive enough to permit an understanding of their relation to the photoreaction which controls flowering.

The low-intensity light reaction appears to inhibit the dark process in the flowering response through effects on the auxin system of the plant. This reaction appears to be made up of a series of partial reactions none of which are completely understood. The equivalence of the primary photoreaction in the various responses seems reasonably well established but a vast amount of additional research is required before the biochemical nature of the reactions subsequent to this primary photoreaction are known. The most profitable approach to this problem would seem to lie in studies directed primarily toward the study of the interaction of auxin and light in simpler systems, with concommitant extension of these findings to the flowering response in both LDP and SDP.

The Second High-Intensity Light Process

In the last section it was shown that a dark period must be preceded by a period of high-intensity light before induction is possible (4, 12). This light reaction apparently supplies building blocks and energy sources for various reactions carried on in the plant in the dark (15). Hamner has observed that flowering is considerably reduced when an inductive dark period is followed by a period of low-intensity light or by continuous darkness (7). Mann (8) and Snyder (9) have confirmed these observations. Recently Lockhart & Hamner (56) confirmed and extended these results and showed that flowering is inhibited when *Xanthium* is given a dark period longer than the critical followed by a flash of light which is then followed by a second dark period. The inhibition resulting from this second dark period is proportional to its

length, and its effectiveness appears to be increased by the addition of auxin or by increasing the temperature (136). This second dark period no longer inhibits flowering if a short period of high intensity light is given between the two dark periods. These results suggested to Lockhart and Hamner that a period of high-intensity light is needed to "stabilize" a flower hormone precursor which they postulate as the primary dark product. They realized, however, that there is no absolute requirement for light since plants will flower in continuous darkness (5, 7).

The fact that high-intensity light prevents inhibition during the second dark period and that the dark reaction appears to have a high temperature coefficient for inactivation of florigen, both suggest a heat-accelerated reaction linked with an energy supplying reaction. If energy, required to keep reactions going toward florigen formation, were in short supply, then raising the temperature would cause a faster dissipation of the energy. Conversely, a period of high-intensity light would prevent destruction of florigen by supplying adenosinetriphosphate (25) directly or by providing substrate, which upon oxidation, would yield energy. Although attempts to prevent inactivation during the second dark period by sugar application have been unsuccessful (11), there is no assurance that the sugar was applied enough in advance to insure its presence in the leaf during the flash and subsequent dark period. The relatively frequent inability to demonstate the inhibitory response to a second dark period may be a function of the amount of photosynthate produced in the previous high-intensity light period. The observed variation in optimal night length may also be a function of the effectiveness of the previous light period. It is not immediately obvious, however, that the ability of plants to flower in continuous darkness is a function of the previous light period since Lang (24) has found with *Xanthium*, that a minimum of at least five days of short cycle treatment (3 hr. dark, 3 min. light) are necessary to prevent flowering in subsequent long periods of continuous darkness. It seems more likely that the energy for flower hormone formation in this instance arises from the slow hydrolysis and subsequent oxidation of less readily available substances in the plant.

An alternate, or possibly a supplementary explanation for the inhibition caused by the light flash followed by darkness, is that this light flash mediates a reaction identical to that initiated by the low-intensity light reaction of the dark process as discussed earlier. If this is true, then this light flash inhibition should be reversed when followed by exposure to far-red. In addition, the light flash should not inhibit in the presence of an antiauxin. The additional inhibition produced by auxin if light is also given supports the latter view (56). This alternative, i.e., that this reaction is similar to the low-intensity light reaction, is in keeping with an earlier suggestion of the author (54, 59) to account for the ability of antiauxin to promote flowering of SDP which are minimally induced. Experiments of Galston (47, 137), Esteves-de-Sousa (138), and the author (54) show that flowering in SDP is markedly increased when antiauxin is applied to plants in which the controls barely

flower. The suggestion (40) is that since florigen appears unstable to auxin until it is "fixed" in some manner by high-intensity light, antiauxins should increase flowering by preventing florigen destruction by the native auxin and light during the day following the dark period. Although both far-red reversibility (83) and antiauxin application (11) have been tried in this respect without consistent success, there appears to be valid reasons for these failures. We now know that far-red radiation, to be significantly effective in reversing red inhibition in *Xanthium*, must be given within 15 to 20 min. of the inhibitory red flash. The few positive results found by Lockhart & Hamner (83) which show a far-red reversal of this red inhibition may be real and their failures due to improper timing between red and far-red exposure. So far as is known, only two unsuccessful attempts have been made to achieve reversal of inhibition by applied antiauxin (11). Again, as in the case with sugar application referred to above, it may be necessary to apply the antiauxin sufficiently in advance of the flash to insure its presence at the site of action when the red flash is given.

A decision as to whether the process herein termed the second high-intensity light process should be considered (*a*) as another variant of the low-intensity light reaction; (*b*) as a process identical to the first high-intensity light process; or (*c*) as an independent, important part of our conceptual framework, must await further critical research along the lines indicated above.

Translocation

Excellent detailed discussions of the general properties of translocation of florigen have been presented recently (1, 2, 40) so that only those aspects reported during the past three years will be given here. Experiments on translocation of the stimulus have been concerned principally with the rate of the process as affected by light and dark and have been performed almost entirely by Salisbury (11) in Bonner's laboratory, and by Khudairi (28) and Lockhart (10) in Hamner's laboratory. The results from the two laboratories are almost identical, however, and since Salisbury's are more extensive they will be discussed here. All experiments were conducted by giving the plants an inductive treatment followed by defoliation at regular intervals after the plants were removed to the long-day greenhouse. An interesting and useful bit of information which comes from these studies is that the rate of translocation is a function of the time of year in which the experiment is conducted. The time from the beginning of a 16-hour dark period until one-half the hormone has moved out of the leaf varies from 24 to 26 hr. in July, 29 to 31 hr. in October or February, and from 40 to 44 hr. in December. Experiments to determine the nature of this difference were carried out in the Earhart Plant Research Laboratory where all the variables except light intensity were controlled. It was found that the changing response during the year is not a function of temperature or humidity, but, apparently, a function of light intensity, i.e., the higher the light intensity, the faster the rate of

translocation. In most cases reported, the minimum amount of time which must elapse before translocation begins is of the order of 20 to 22 hr. from the beginning of the dark period. It is an interesting fact that this time interval before translocation begins bears no obvious relation to the length of the dark period preceding the exposure to light. Apparently, then, we are confronted with an interesting paradox. On the one hand, the rate of translocation of florigen out of the leaf appears to be a function of light intensity. On the other hand, plants receiving night lengths of 10 to 20 hr. in length do not begin translocation of florigen until some 22 hr. after the beginning of the inductive dark period. Thus, plants receiving a 10-, 14-, 16-, or 20-hour night would receive respectively 12, 8, 6, or 2 hr. of high-intensity light before translocation begins. Further vigorous studies are needed on the kinetics of the process of translocation of florigen, particularly regarding the relation between the movement of florigen and the movement of photosynthates following exposure of the plants to different night lengths. These studies promise to reveal to us more of the nature of florigen and its formation and stability under various conditions.

Differentiation

Once the flowering hormone has reached the growing bud, differentiation begins almost immediately. This view is supported by the fact that translocation out of the leaf does not begin until some 20 hr. from the beginning of the dark period (11), and the first visible anatomical changes begin to occur some 40 to 60 hr. later (11, 139). Although the exact nature of these changes is not clear, we do know that an active bud or its equivalent is necessary (11, 69, 70, 75). Young leaves (75) or added auxin (11) are able to replace the need for an active bud for a few days (see p. 186). In addition, auxin promotes flowering even in the presence of an active bud (11), possibly by helping florigen bring about faster differentiation of the terminal bud. The rate of differentiation apparently depends upon the quantity of florigen which reaches the tip since more florigen (longer induction) increases bud differentiation rate up to a saturation level. Above this point, additional florigen apparently has no effect, presumably because some other reactant becomes rate limiting.

What do we know about the chemical nature of the substance which brings about the rapid biochemical changes connected with the initiation of flowers, i.e., which changes a vegetative plant to a flowering plant? Unfortunately, we know very little about this postulated substance. Until further evidence concerning its chemical nature has been demonstrated, the author still agrees with the suggestion of Bonner & Liverman (2) that it may be a substance of relatively high molecular weight, possibly a protein with virus-like properties.

During the past three years, additional grafting experiments by Khudairi & Lang have further indicated that the flowering hormones of both LDP and SDP are identical (24, 140, 141). They showed that plants of Maryland

Mammoth tobacco, a SDP, kept vegetative by exposure to long days, flowers only when the LDP donor to which it is grafted (*Hyoscyamus*) is placed in an inductive day length. A Maryland Mammoth donor placed in inductive day lengths causes flowering of a vegetative *Hyoscyamus* plant only when the tobacco plant has from 5 to 10 leaves. With *Hyoscyamus* as the donor, only a single leaf is required. It appears that Maryland Mammoth produces relatively less flowering hormone than does *Hyoscyamus*. It is apparent then, that in the flowering reaction we are dealing with the ultimate synthesis of a single active substance both in LDP and in SDP. The difference between the two types appears to reside in their respective response to the level of some substance, possibly auxin. This matter will be discussed in more detail in the final section.

PROPOSALS CONCERNING THE MECHANISM

In the foregoing discussion, we have considered new data on flowering and related responses accumulated during the past three years. A discussion of work with inductive cycles longer than 24 hr. has purposely been omitted in order to keep the picture as simple as possible. The facts suggest strongly the conclusion that the reactions leading to floral induction are, in general, quite similar if not identical in both LDP and SDP. The difference in response to the stimuli of long nights and of low-intensity light appears to be more of a degree than of kind. A division of the photoperiodic response into several partial processes has been made to serve as a framework for comparison of the responses of LDP and SDP. Let us now review these facts and try to integrate them into a working hypothesis as a help in planning future research. For the sake of clarity this discussion will principally concern flowering of SDP and only incidentally that of LDP. Since the proposals made here are to serve only as a working hypothesis for the organization of our thinking concerning the photoperiodic mechanism, they are, of necessity, quite tentative and will have to be modified as new facts become available.

High-intensity light.—A multiplicity of photochemical reactions take place in the mature green plant as a result of exposure to high-intensity light during the day in the greenhouse. The most important of these reactions is the photosynthetic fixation of CO_2. The carbon from this CO_2 finds its way into hundreds of compounds in the plant at least two of which are directly concerned with the inductive process, (*a*) sugar or other respiratory substrate which can be utilized for energy or for building blocks, and (*b*) auxin. Other photochemical reactions which take place during the first high-intensity light period and which are of importance to the inductive process are (*a*) a shift in equilibrium of the red-, far-red light receptor pigment system to the far-red absorbing form, and (*b*) a possible activation of IAA oxidase. We find, then, at the end of a day in the greenhouse that the concentration of sugars, of auxin, and of the far-red absorbing form of the pigment is high.

Uninterrupted dark period.—When the plant is moved to darkness a variety of reactions begin to take place. The pigment system begins to revert

back to the red absorbing form. This reaction goes to completion in 2 or 3 hr. as indicated by three lines of evidence. First, exposure to far-red radiation during the last 30 min. of the light period shortens the critical dark period by some two hr. (22). Second, the light energy required to achieve the maximum red light effect decreases up to 2 to 3 hr. and is relatively constant thereafter (11). Third, auxin is maximally effective in causing inhibition if applied about 2 or 3 hr. after the beginning of the dark period (11).

In addition to the shift in the pigment system there would appear to be a continuing decrease in the leaf auxin level in the dark. Auxin in this sense includes both the free auxin and the growth active auxin-receptor complex. Although it has never been shown conclusively that the auxin level does fluctuate in the plant in a manner correlated with the photoperiodic response, there is evidence that plants grown under long-day conditions contain more auxin than those grown under short-day conditions (2). These results coupled with those which show that the auxin inhibition of flowering is relatively specific for auxins, suggest that once the auxin reaches some critically low level, there is a net appearance of florigen in the leaf. The fact that auxins continue to inhibit flowering well into the light period following the dark period leads one to suspect that auxin in some way causes an inactivation of the florigen molecule itself. This conclusion is supported by the investigations on red light interruption of the dark period. A red light flash given after the critical dark length appears to stop florigen production. A red light flash, plus auxin, causes florigen disappearance. Possibly auxin controls this response by mediating the utilization of ATP. Thus, if ATP is required to synthesize and maintain the florigen molecule intact, high auxin levels would inhibit flowering by diverting ATP away from maintaining the equilibrium in the direction of florigen synthesis and toward the pumping of water into the cells, making leaf protein precursors, etc. Once begun, the synthesis of florigen continues unabated until the optimum night length is reached. If light is not given at this time, flowering is gradually reduced, with the rate of this reduction being dependent upon the physiological age of the leaf and possibly also upon the availability of energy-yielding substrates. One suggestion as to the possible nature of this synthesis comes from experiments (37) which show that exclusion of CO_2 from SDP during the dark period prevents induction.

In *Xanthium* flowering is a function of the length of the dark period in excess of 8.5 hr. and most of the evidence currently available suggests that florigen synthesis stops as soon as the dark period is ended. Other interpretations of this data are possible, but this appears to be the simplest. The facts that auxin continues to inhibit flowering well into the next light period (11, 40) and that antiauxins increase flowering of minimally induced plants (40, 47, 138) appear to be attributable to the destruction of florigen by auxin.

Interrupted dark period.—The reactions which go on during a dark period are also inhibited by a flash of red light given at the appropriate time during the dark period. The time of maximum effectiveness is in the region of 8 hr.

after the beginning of the dark period (11). This light flash inhibition apparently is intimately related to the auxin metabolic system, since both the inhibition produced by light and by auxin are reversed by antiauxin (40, 49, 54). In the case of LDP, a dark period inhibits flowering and applied auxin or red light promote flowering so that the same basic mechanism appears to be operative (78). This view is further strengthened by the observation that far-red radiation reverses both the promotive light effect in LDP and the inhibitory effect in SDP (22).

These results may be understood on the basis of the photocyclic concept of Liverman & Bonner (26) which has been presented in detail on page 180. According to this concept, the active auxin, i.e., the auxin-receptor complex, *ES*, is the important factor in controlling various morphological responses such as flowering. Red light affects the formation of this complex by causing the conversion of an inactive auxin receptor, E_p, into the active receptor *E*. *E* then combines with the free auxin, *S*, to form the growth active auxin-receptor complex, *ES*. Far-red negates the red-promoted reaction by causing the decomposition of this complex into E_p and *S*. Thus, red light would inhibit flowering of SDP and promote flowering of LDP by causing the formation of more *ES*. This added amount of *ES* puts *ES* in the inhibitory range for SDP and in the promotive range for LDP.

Why the red light should be more effective at approximately 8 hr. after the beginning of the dark period is purely conjectural (11). It may be, however, that the effectiveness of light at this time is related in some way to Bünning's "endogenous rhythm." That rhythmic responses do, in fact, exist has been known for a long time and has been amply demonstrated by Bünning (142, 143) and others. The unfortunate aspect of Bünning's theory is that it offers no specific suggestions on experimental approaches to learning the nature of the flowering response and this fact has caused it to be almost completely ignored in the United States. To the author its chief value appears to lie in the fact that it rather accurately predicts the times at which light will be most effective in inhibiting or promoting flowering. The fact that this theory does predict such occurrences makes it imperative that one consider it in formulating any working hypothesis concerned with flowering. Fortunately, Galston (128) has recently provided us with information concerning the auxin metabolic system which may put biochemical meaning into Bünning's theory. Thus, Galston has observed that IAA oxidase is adaptively formed in response to applied auxin but not in response to compounds such as benzoic acid which is inactive as an auxin. Although Galston has not demonstrated the de-adaptive disappearance of the oxidase upon withdrawal of the specific substrate, this would seem to be likely. Galston has proposed that this sytem of adaptive formation and de-adaptive disappearance of the oxidase acts together with the auxin synthetic system to determine the auxin level in the plant. Thus, as the auxin level starts to rise, adaptive formation of IAA oxidase begins. As long as the rate of synthesis exceeds the rate of destruction, the free auxin level rises, but as forma-

tion of the oxidase increases, a level is reached where destruction equals synthesis. From this point on, the auxin level decreases because the rate of destruction is greater than that of synthesis. As the auxin level is reduced, the activity of the oxidase decreases until destruction and synthesis are again equal. From this point on, auxin synthesis exceeds destruction and the cycle begins all over again. The cycle will continue in darkness until auxin precursors become limiting and the amplitude of the fluctuations are reduced below the point of detection of the rhythm. The application of sugar increases the amplitude of flower petal movement (144) and it could also serve as an early precursor of auxin.

The biochemical mechanism proposed by Galston (128) as a basis for Bunning's endogenous rhythmicity, coupled with the photocyclic concept of light action proposed by Liverman & Bonner (26) allows us to encompass most of the observations of the interaction of light and auxin in the flowering response of both LDP and SDP within one conceptual framework. In part, these concepts are illustrated graphically in Figure 2.

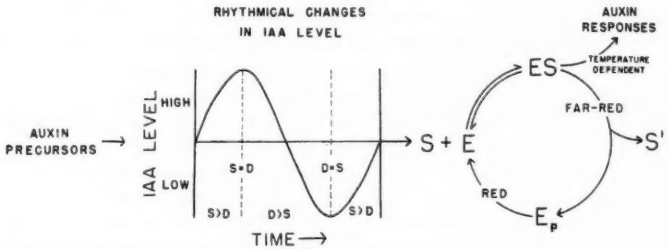

FIG. 2. Proposals concerning the photoperiodic mechanism. See text for details. Left portion of figure modified from Galston *et al.* (128). Right portion modified from Liverman & Bonner (26).

Thus, as outlined earlier, the high-intensity light process is characterized in part by the formation of auxin precursors. The net concentration of auxin (S) depends on the balance between its synthesis and its destruction by the adaptive formation and the de-adaptive disappearance of IAA oxidase. The suggestion that the auxin level decreases during the dark concerns both forms (free S and ES) but principally ES since this is the form responsible for various auxin-mediated reactions. The level of ES can be increased by adding S exogenously or by causing a formation of more E from E_p by exposure to light. A flash of light would be most effective in mediating the red light reaction if given when the endogenous level of S is high and E is principally in the form of E_p. These relations suggest, superficially at least, that light should be effective at any time during the dark period if auxin is applied sufficiently in advance to be present at the site of action when the flash of light is given. Few, if any, experiments have been carried out to determine

if this is correct. The author in cooperation with Lang (78) has found, however, that light and auxin both of which alone were inadequate to cause floral induction, caused up to 100 per cent of the treated plants to flower if applied together to the leaves of *Hyoscyamus*. The author (40) has obtained a similar effect of auxin and light in the inhibition of flowering of SDP. The experiments were carried out in both cases under constant light of low intensity during the dark period. Salisbury (11) has reported results which, on the surface, appear to contradict this conclusion. It is not evident, however, that the necessary precautions were taken to insure that auxin was present at the site of action at the time the flash was given.

The exact relation between the pigment system of Borthwick and his co-workers, which is responsible for perception of the light and E_p, E, and ES is not clear. Possibly Borthwick's pigment is identical to the Todd-Galston pigment and the pigment functions by being an integral part of the E molecule. It is equally possible, of course, that the light receptor is an entirely separate entity which is in constant collision with E_p and ES in the cell. The fact that auxin must be present for the reaction to be reversed by far-red light suggests the first alternative. That other enzyme systems also appear to be affected by this same light reaction suggests the latter possibility. It is not yet possible to choose between these alternatives or, indeed, to say whether either is correct. In any case, once the level of ES has been increased it proceeds to redirect the utilization of ATP into other channels. This portion of the reaction sequence is evidently temperature-dependent since low temperature decreases the growth rate and the rate of the inductive processes in SDP. This is in keeping with the findings of Borthwick, Hendricks and Parker. The biochemical nature of these further reactions is unknown. They do appear to involve the utilization of CO_2, but a complete understanding of this aspect of the problem and of the actual biochemical pathways involved in the synthesis of florigen once the ES level is sufficiently low, must await future developments.

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PHYSIOLOGY OF ABSCISSION[1,2]

By Fredrick T. Addicott and Ruth Stocking Lynch
Department of Botany, University of California, Los Angeles, California

Introduction

Abscission, the detachment of a plant organ or other part, has long held the interest of botanists [Mohl (88); Wiesner (139)]. The early work was chiefly descriptive. Extensive morphological studies have been published [e.g., Lee (68); Pfeiffer (99); Sampson (106)]; some of these include speculation on the regulation of abscission [Facey (38); Goodspeed & Kendall (45); Sampson (106)]. Study of the process was stimulated in 1939 when field control of abscission was initiated [Gardner *et al.* (42)]. In recent years an increasing number of physiological publications have appeared.

This review covers the subject rather than the literature of abscission physiology. References have been selected for priority, comprehensiveness, or other special quality. The morphology and agricultural regulation of abscission are outlined.

The recently proposed nomenclature of chemical plant regulators [Overbeek (96)] is followed. The convenient term "explant" is used to designate an excised leaf abscission zone [Addicott *et al.* (4)].

Morphology

Common examples of abscission are the detachment of leaves, of flower parts, and of fruits and seeds. Other examples are the shedding of bark and fruit hulls and the dehiscence of anthers and fruits. Understanding of abscission has been greatly advanced by morphological investigations which have defined the regions and tissues involved and have focused attention on the critical phase—cell wall dissolution.

Involved in the abscission of most organs is a discrete abscission zone located at the base of the organ. The abscission zone is often constricted relative to contiguous regions. Its cell walls are thin, completely or nearly lacking in lignin and suberin [Scott *et al.* (107)]. It is considered a region of arrested maturation, its principal activities beginning with the senescence or injury of the organ it subtends. How senescence or injury of the organ stimulates the activity of a thin disc of cells at its base (the abscission zone) is one of the unsolved problems of physiology.

A series of cell divisions usually precedes separation. Separation without cell division occurs in leaf abscission of certain species, in ethylene-induced leaf abscission of other species [Gawadi & Avery (44)], in anther dehiscence

[1] The survey of literature pertaining to this review was completed in October, 1954.

[2] For abbreviations which appear in the text, see list appended to Table II, page 230.

[Esau (36)], and in some types of fruit dehiscence [Rethke (103)]. This indicates that cell division is not an essential precursor of separation; its function appears to be the development of the protective layers of the abscission scar [Gawadi & Avery (44); Lloyd (73)].

Separation may result from the dissolution of one or more layers of cells or cell parts. Three types of dissolution have been observed [Addicott (1); Pfeiffer (99)]. In the first type the middle lamella between two layers of cells dissolves, the primary cell walls remaining intact. In the second type both middle lamella and primary cell walls between two layers of cells dissolve, leaving, at most, thin cellulosic walls over the protoplasts [Eames & MacDaniels (31)]. In the third type entire cells of one or more layers dissolve. The existence of this third type is in dispute [Lloyd (74)]. In such types, dissolution can be considered the key reaction in the physiology of abscission. In other cases no dissolution occurs; abscission appears to be effected by physical stresses, as in the leaf abscission of most monocotyledons and herbaceous dicotyledons [Eames & MacDaniels (31); Pfeiffer (99)]. In some, tissue tensions determine the pattern of abscission [Brown & Addicott (18); Livingston (72)]. The dehiscence of capsules and legumes results from tensions developing with desiccation of the fruit wall. The force for abscission of the fruit cap of the pyxidium is provided by growth of the seeds [Rethke (103)].

Physiology

General.—A healthy organ remains attached indefinitely. Typically, senescence or injury precedes abscission: fruits ripen, leaves yellow before they fall, and cells of ovulary walls die before or with dehiscence [Rethke (103)]. Leaves that are injured fall. In *Citrus*, truncated leaves fall at rates directly proportional to the area removed [Livingtson (72)], although in beans more than 75 per cent of the blade must be removed before the attached portion shows an accelerated abscission [Swets & Addicott (116)]. Infection by certain microorganisms, feeding by certain insects, injury by many types of chemicals, are followed by accelerated abscission. In some species physical injury may be followed within minutes by corolla abscission [Kendall (63)]. When conditions prevent development, immature organs, such as vegetative buds, flower buds, and young fruit, may abscise. A number of preabscission changes may take place in leaves: development of anthocyanin; disappearance of chlorophyll; exit of nitrogen, phosphorus, potassium, iron, and magnesium; change in form or disappearance of carbohydrates or both; decrease in moisture; and decrease in auxin [Hall & Lane (55); Miller (85); Shoji *et al.* (110)]. These observations indicate that the factors which regulate abscission lie chiefly within the organ concerned.

But factors external to the organ also affect its abscission. In deciduous fruit trees the amount of carbohydrate stored in the tree largely determines the amount of abscission of young fruit (June drop) [Chandler (26)]. In certain evergreen trees (e.g., avocado, camphor tree, *Citrus*), appearance of

flowers or new leaves in the spring is followed by a flush of leaf abscission [Cameron *et al.* (21); Wiesner (141)]. Environmental factors such as temperature, water, mineral nutrients, and photoperiod also affect abscission, probably indirectly through their effects on the organ or organism concerned.

In many species, pollination, fertilization, and embryo development affect the abscission of flower parts. In *Clarkia*, for example, the petals abscise shortly after fertilization, the latter occurring about 40 hr. after pollination; if fertilization does not occur the petals remain attached for many days. Further, if embryos fail to develop, the ovulary is abscised; if they do develop, the ovulary remains attached until maturity [Lewis (71)]. Horticulturists recognize three waves of abscission of young fruit of deciduous fruit trees: the first, at blooming time or shortly after, following pistil abortion; the second, about two weeks after bloom, following failure of fertilization; and the third, June drop, following competition for nutrients and failure of embryo development [Chandler (26)]. The factors affecting abscission are summarized in Table I.

Auxin.—In 1933 Laibach (66) observed that application of orchid pollinia (a source of auxin) retarded abscission of debladed petioles. This was confirmed by LaRue (67) working with pure IAA (indoleacetic acid) and repeatedly confirmed since then [Addicott & Lynch (2); Gardner & Cooper (41); Myers (91); Wetmore & Jacobs (137)]. Auxin retarded abscission in explants, when applied to the entire explant by immersion [Livingston (72)], or to the distal side of the explant in droplets (Fig. 1) [Addicott & Lynch (2)] or agar blocks [Addicott *et al.* (6); Louie & Addicott (76)]. Application of IAA and related regulators retarded abscission of leaves, flower parts, young fruit, and mature fruit of many species (see subsequent section on Agricultural Regulation of Abscission). In a number of species and varieties, however, abscission was not retarded by auxin [Batjer (12); Vyvyan (122); Wester & Marth (135)].

Several studies of endogenous auxin in relation to abscission have been made. In bean leaflets a decrease of free auxin immediately preceded abscission (Fig. 2) [Shoji *et al.* (110)]. In *Coleus*, as the leaves matured and approached abscission, a gradual decrease in diffusible auxin occurred [Wetmore & Jacobs (137)]. Other studies showed a similar decrease in diffusible auxin with maturation of leaves [Avery (9); Goodwin (47); Went & Thimann (134)]. In the development of apples, periods of high auxin production by the seeds are correlated with periods of negligible fruit abscission [Luckwill (77)]. In bean and cotton the gradient of auxin across the abscission zone fell as abscission approached. Shoji *et al.* (110) found the gradient in fully mature bean leaves was three μg./kg. fresh weight in the leaflet, distal to the abscission zone and one μg./kg. fresh weight in the stalk, proximal to the abscission zone (Fig. 2). Shortly before abscission the gradient disappeared. In a similar investigation with cotton the gradient decreased immediately preceding leaf abscission [Carns (22)].

Auxin, under certain conditions, may accelerate abscission. Application

TABLE I

FACTORS KNOWN TO AFFECT ABSCISSION

Required:	Oxygen Respiration Water	Moderate temperature Healthy abscission zone
Inhibiting or retarding		Initiating or accelerating
Presence of the organ in a healthy condition (leaf blade, ovulary, etc.)		Senescence, disease, injury, or removal of the organ
Embryo development (inhibits ovulary abscission)		Embryo development (initiates petal abscission)
Auxins		Auxin (applied proximal to the abscission zone)
		Antiauxins (triiodobenzoic acid, maleimides, etc.)
Balance of nitrogen and carbohydrates		Extreme imbalance of nitrogen and carbohydrates
		Mineral nutrient deficiency (especially Ca, Mg, S, and Zn)
Water (applied to explants)		Drought
		High Humidity
		Flooding
		Extremes of temperature (especially frost)
Long, or uniform photoperiod		Shortening photoperiod
		Oxygen (above 20 per cent, applied to explants)
Carbon dioxide (applied to explants)		Carbon dioxide (applied to flowers)
		Unsaturated hydrocarbons (acetylene, butynediol, ethylene, ethylene chlorohydrin, etc.)
		Defoliants (aminotriazole, chlorates, copper salts, cyanamides, endothal, ethyl xanthates, thiocyanates, etc.)

of IAA to the proximal side of an abscission zone accelerated abscission in explants of bean (Fig. 1) and cotton [Addicott & Lynch (2); Louie & Addicott (76)] and in debladed leaflet pulvini of bean plants in the greenhouse [Swets & Addicott (116)]. In the explants the maximum rate of abscission was equal to the rates produced by ethylene, oxygen, and other potent accelerants. Although unexpected, such results were not surprising since auxin is known to accelerate or retard other processes (e.g., growth, flowering) depending on concentration and other factors, and application of auxin-like growth regulators has been observed to accelerate abscission of cut flowers. However, in many species and varieties attempts to regulate abscission by application of auxin-like growth regulators have been unsuccessful [Vyvyan (122); Wester & Marth (135)].

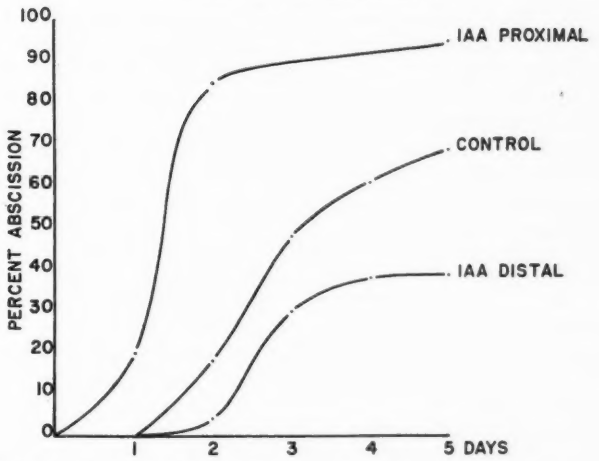

FIG. 1. Effect of the site of IAA application on abscission in bean explants. Illustrated is the acceleration of abscission by application proximal to the abscission zone and retardation of abscission by application distal to the abscission zone, [after Addicott & Lynch (2)].

Two aspects of the above results are worthy of further comment. First, the action of auxin depends on the site of its application. Growth substances and nutrients are transported preferentially to regions where auxin is in relatively high concentration (e.g., meristems, young fruits) [Emsweller &

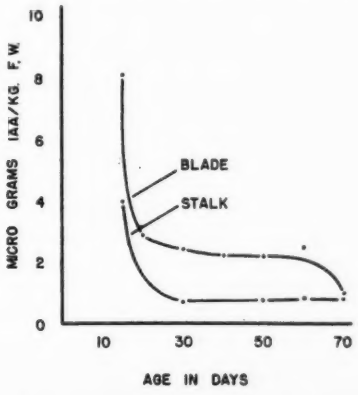

FIG. 2. Changes of auxin concentration in bean leaf blades and leaf stalks with age. At 70 days the blades were yellow and about to fall, [after Shoji *et al.* (110)].

Stuart (35); Went (131)]. Hence, augmenting the auxin supply distal to an abscission zone (in a fruit, leaf, or petiole) should help to maintain the flow of nutrients to the organ and thus indirectly retard abscission. The additional auxin might also directly retard abscission. Conversely, application of auxin proximal to the abscission zone should accelerate the preabscission withdrawal of nutrients and thus indirectly accelerate abscission. Second, there is a diversity of effect (retardation, acceleration, or no effect) following application of growth regulators to intact plants of various species. Such diversity is not surprising in view of the known differences among species and the complicated nature of auxin physiology. Plants differ considerably in ability to absorb, transport [Mitchell & Marth (87)], and inactivate auxins, as well as in amounts of endogeneous auxin and inhibitors. Failure to respond might result from one or more of these factors: failure of absorption, failure of transport, types of absorption and transport that maintain the existing auxin gradient, or rapid inactivation. Retardation probably results from augmentation of existing auxin gradients. Acceleration might result from types of absorption and transport that lower the auxin gradient across the abscission zone, or possibly from competitive inhibition of auxin in the organ concerned. The latter possibility appears unlikely since application of IAA (25 to 500,000 μg./l.) to the distal portions of cotton explants produced increasing retardation with increasing concentration and no sign of reversal [Addicott *et al.* (6)].

Auxin relations of other factors will be discussed in other sections. For want of a more appropriate place it is mentioned here that the fungus *Omphalia*, whose infection of coffee leaves produces considerable defoliation, appears to act by the production of an auxin-inactivating enzyme [Sequiera & Steeves (108)].

Nitrogen and other mineral nutrients.—The effects of the carbohydrate-nitrogen ratio on growth and fruitfulness are paralleled by its effects on abscission. Nitrogen retards and reduces abscission. Under conditions of ample nitrogen, leaves are retained longer and fewer flower buds and fruit are shed [Heinicke (58)]. Conversely, nitrogen deficiency increases abscission [Chandler (26); Nightingale & Farnham (92)]. Obviously, nitrogen is required for auxin synthesis. The amount of nitrogen and other mineral nutrients is correlated with auxin concentration [Avery *et al.* (10); Avery & Pottorf (11)]. Calcium-deficient *Citrus* leaves abscise prematurely [Chapman & Kelley (27)]. Calcium pectate is the important constituent of the middle lamellae holding together the cells of the abscission zone. Prior to abscission the middle lamellae lose considerable calcium [Sampson (106)]. Zinc deficiency leads to premature leaf abscission, and zinc is specifically required for auxin synthesis [Skoog (111)]. Deficiency of sulfur and magnesium also leads to leaf abscission [Bear *et al.* (14); Chapman & Kelley (27)], but the physiology of their effects on abscission is unknown.

Carbohydrates.—Low carbohydrates lead to leaf, flower bud, and fruit abscission [Chandler (26); Nightingale & Farnham (92)] and favors defolia-

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tion [Hall (51)]. Competition for carbohydrates underlies the June drop of young fruit of deciduous fruit trees; this abscission can be reduced by ringing, which increases carbohydrates above the ring [Chandler (26)]. Application of sucrose has retarded or reduced abscission [Brown & Addicott (18); Heinicke (58); Went & Carter (132)]. Moderate nitrogen [Nightingale & Farnham (92)], low water supply, high light intensity [Mason (80)], and, in general conditions which favor carbohydrate accumulation are accompanied by reduced or retarded abscission.

Carbohydrates can affect abscission in at least two ways: (*a*) increased carbohydrates lead to thicker cell walls making abscission more difficult; and (*b*) transport of carbohydrates to ovularies supports embryo development; embryos produce auxin which in turn retards abscission.

In addition to these general effects, carbohydrates in the abscission zone show a direct relation with abscission. Morphologists frequently find larger deposits of starch in the abscission zone than in contiguous regions [Griesel (48); Lee (68); Livingston (72)]. In some species starch deposits are present before abscission, disappearing during abscission; in other species, they are absent before abscission, appearing during abscission. For example, in *Magnolia* perianth abscission, starch is present in the abscission zone before abscission, disappears during abscission, and after abscission, dextrin-like granules appear on the proximal side of the abscission zone [Griesel (48)]. Nothing is known of the regulation of this carbohydrate behavior.

Oxygen, carbon dioxide, and respiration.—Reduced oxygen tensions have retarded abscission [Molisch (89); Sampson (106)]. Bean leaflet explants did not abscise when kept in water, mineral oil, or nitrogen gas, but did abscise in water through which oxygen was bubbled. In a moist atmosphere the rate of abscission of explants was correlated with oxygen concentration from 0 to 50 per cent; above 50 per cent oxygen there was no further increase in rate (Fig. 3). The maximum rate of abscission in oxygen was greater than the rate in air and equivalent to the rate produced by ethylene [Carns *et al.* (25)]. Previous to these observations the oxygen acceleration of abscission was unknown.

Complete respiration may be essential to abscission. Selected for diversity of effect on respiration, solutions of seven enzyme inhibitors were applied to explants. Abscission was retarded by all and, with one exception, at all concentrations; it was slightly accelerated by iodoacetate at 10^{-5} *M* [Carns & Addicott (24)]. Abscission's requirement of the complete process of respiration may be the main basis for its sensitivity to so many types of substances (see list of retardants and accelerants in Table II). A climacteric rise in rate of respiration coincident with abscission was disclosed by further study [Carns (23)]. Yet to be elucidated are the physiology of oxygen acceleration and the role of respiration in abscission. Oxygen acceleration may be the result of inactivation of auxin, since inactivation is an oxidative process [Tang & Bonner (117)].

Carbon dioxide accelerated flower abscission in *Nicotiana;* 10 per cent

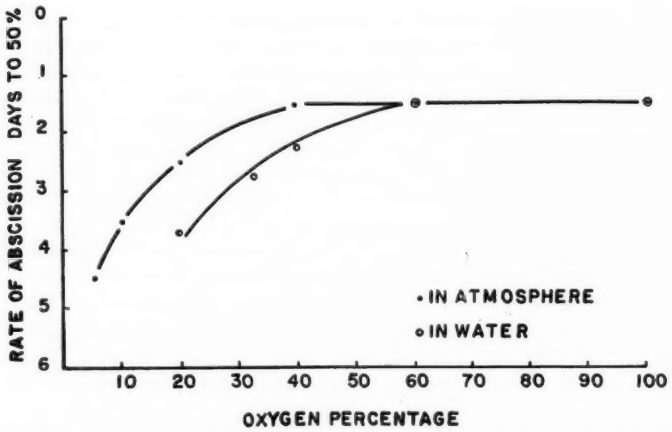

FIG. 3. The effect of oxygen on abscission in bean explants with the explants held in gas mixtures or submerged in water, [after Carns *et al.* (25)].

carbon dioxide reduced the period required for flower fall from 34 hr. to 17 hr. [Goodspeed *et al.* (46)]. Carbon dioxide also retarded abscission; 5 to 22.5 per cent carbon dioxide, in oxygen, retarded abscission in bean leaflet explants. The retardation was small at 5 per cent carbon dioxide, slowly increased as the carbon dioxide increased to 17.5 per cent, rapidly increased above 17.5 per cent. In an unconfirmed experiment, 11 p.p.m. ethylene added to carbon dioxide-oxygen mixtures prevented retardation by the 5 per cent carbon dioxide mixture, and greatly reduced retardation by the 20 per cent carbon dioxide mixture [Yamaguchi (147)].

Enzymes.—Oxidases have been found in all tissues of the abscission zone except the xylem, although in contiguous regions they occur only in the epidermis and phloem. Oxidase activity increases with age of the abscission zone [Sampson (106)]. Catalase was found more active in the abscission zone than in contiguous regions [Heinicke (58)]. Aside from the work of Carns with inhibitors of respiratory enzymes (see preceding section), further investigations of enzymes and abscission have not been made. The morphological evidence suggests that enzymes are directly involved in the cytolytic phase of abscission. Pectic compounds, and often cellulose and lignin, are digested during abscission [Pfeiffer (99)]. With one possible exception [Kertesz (64)] these substances are digested *in vivo* only in the presence of enzymes. The speed of abscission also suggests enzymatic activity; only seconds or minutes are required for certain types of corolla abscission [Kendell (63)]. What initiates cytolytic enzyme activity is unknown. The respiration requirement suggests that the first step is synthesis of the enzymes. On the other hand, it is possible that no synthesis occurs and that existing enzymes are activated through some change in the abscission zone.

Acidity.—There is very little evidence on the function of pH in abscission; the few workers who have considered the subject disagree. Wiesner (139, 143) found that (*a*) yellow leaves contained more titratable acid than green leaves, (*b*) accelerated leaf abscission followed placement of cuttings in 2.5 per cent oxalic acid, and (*c*) the exposed abscission surface of a petiole always gave an acid reaction to neutral red. On this evidence he based a theory that abscission is the result of the accumulation of organic acids in the abscission zone. Sampson (106) found that *Coleus* leaves contained less acid during abscission than before it, and that oxalic acid did not accelerate abscission except in concentrations which first produced toxic reactions. He pointed out that free pectic acid is to be expected on exposed abscission surfaces and that its presence there does not necessarily support the view that abscission is caused by the accumulation of organic acids in the abscission zone. Facey (38), using *Fraxinus*, extended certain of Sampson's experiments. She placed cuttings in various concentrations of hydrochloric acid and exposed them to various concentrations of ammonia vapor. With both substances only those concentrations which first produced a toxic reaction accelerated leaf abscission. Low concentrations of ammonia slightly retarded abscission. Certain intermediate concentrations of hydrochloric acid led to a partial breakdown of pectic materials but did not accelerate abscission. Facey assumed that these treatments affected the pH of the leaf abscission zone and concluded that her results supported Wiesner's idea that a change in acidity in the cells of the abscission zone is the causative factor in abscission. The reviewers feel that much more information must be assembled before the relation between pH and abscission can be understood.

Temperature.—Several observations have been made of the effect of temperature on abscission. In general, the effects are similar to the effects on other physiological processes. For example, in bean leaflet explants the rate of abscission is very low under 5°C., rises with temperature to a maximum between 25° and 30°C., and falls at higher temperatures [Yamaguchi (147)].

There are also a number of indirect effects. Exposure to relatively extreme temperatures is often followed by increased abscission of leaves, flowers, or fruits [Chandler (26)]. In most if not all of these cases, not the temperature directly, but some intermediate factor, such as injury, limited translocation of foods, or high rate of respiration, is probably responsible for the abscission [Chandler (26); Meyer & Anderson (83); Went & Hull (133); Yamaguchi (147)]. An interesting example of low temperature effect is found in cotton leaf abscission. If the first frost is relatively light, leaf abscission is accelerated and defoliation results. If the first frost is relatively heavy, the leaves remain attached. The light frost injures the leaf blades without seriously affecting the abscission zones or the stem, and leaf abscission proceeds as is usual after a blade injury. The heavy frost kills the entire plant, preventing abscission.

Water.—Abscission is affected by water in many ways. Water must be readily available if the cells of the abscission zone are to function. Rapid

desiccation may lead to death before abscission can be completed. On the other hand an excess of water around explants retards or prevents abscission [Livingston (72)] by interfering with accession of oxygen [Carns *et al.* (25)]. An excess of water in the soil increases abscission of leaves and fruits [Mason (80)] apparently by interfering with the absorption of mineral nutrients.

Tissue tensions (turgor) in the abscission region may determine the pattern of abscission and may also facilitate separation [Brown & Addicott (18); Livingston (72); Loewi (75)]. Dehiscence of capsules and legumes results from tensions created by the desiccation of dead or dying ovulary walls [Rethke (103)].

During and immediately after abscission the tissues distal to the abscission zone usually lose water rapidly [Brown & Addicott (18)], while the proximal tissues retain moisture and turgor. Careful handling and examination of the proximal tissues discloses a surface covered with turgid cells, clavate or papillate in shape [Brown & Addicott (18); Kendall (63); Ramsdell (102); Scott *et al.* (107)]. These cells become the primary protective layer of the scar.

In greenhouse and field, water deficiency may lead to abscission of leaves, flowers, and fruits [Wiesner (142)]. In certain species (e.g., *Citrus*) appreciable leaf fall from water-deficient plants may not occur until water has been applied; then the fall is rapid. It appears probable that this abscission is initiated in response to the injury of water stress but cannot be completed in the absence of adequate water. Under moisture stress the leaf abscission zone is apparently unable to successfully compete with the rest of the plant for water and cannot obtain enough for the hydrolysis of its cell walls. Application of water permits the stems to regain full turgor, further facilitating the shed of the partially desiccated leaves.

Photoperiod.—The autumnal leaf abscission of deciduous trees is correlated with a shortening photoperiod [Wiesner (140)]. Lengthening photoperiod delayed the onset of leaf abscission [Garner & Allard (43); Matzke (81)]. Shortening photoperiod was the principal factor initiating autumnal leaf abscission in the sugar maple. Further, maple seedlings held under constant photoperiods (8, 12, or 16 hr.) showed considerably delayed abscission; the leaves eventually abscised according to age. The experimental conditions suggested that the photoperiod effect was indirect, and through effects on hormones rather than through effects on nutrients [Olmstead (94)].

Other factors.—Some of the retardants discussed in the previous sections have important practical use. This is also true of NAA (naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), ClPA (*para*-chlorophenoxyacetic acid), and their derivatives (see section on *Agricultural Regulation of Abscission*). Since these are auxins in the broad sense of the term [Overbeek (96)] it is generally held that they retard abscission by augmenting endogenous auxin.

Abscission accelerants are diverse chemically and physiologically. In abscission they are similar in killing or injuring certain organs, thus accelerat-

ing organ abscission without seriously affecting the rest of the plant. For convenience, accelerants will be discussed in five groups: unsaturated hydrocarbons, antiauxins, enzyme inhibitors, defoliants, and fruit thinners.

Among the unsaturated hydrocarbons, ethylene is the most potent accelerator of abscission; it also has several other physiological effects [Crocker (28)]. Only one derivative, ethylene chlorohydrin, appears to affect (accelerate) abscission [Gawadi & Avery (44)]; other derivatives are inactive. Although the general effects of ethylene are well known, and certain specific effects on growth and respiration have been studied, little is known of its biochemical action. Abscission induced by ethylene or commercial defoliants was accompanied by a number of chemical changes in leaves. These included disappearance of chlorophyll, increased respiration, and decreased carbohydrate and nitrogenous compounds [Hall & Lane (55)]. These changes are similar to those accompanying natural abscission as described above. Hall's conclusion that depletion of leaf carbohydrate and nitrogeneous compounds has an important role in the chain of events leading to abscission may be sound; but since abscission proceeds normally in explants, removed from leaf blade and petiole, and the abscission of mature fruits is unaffected by the presence of considerable nutrients in the fruit, it appears more probable that depletion is a side reaction. The possible role of ethylene as an endogenous regulator of abscission is discussed under hypotheses and theories.

Acetylene accelerates abscission [Richter (104)] and has other effects (e.g. epinasty, and root induction) paralleling those of ethylene [Crocker (28)]. The acetylene analogue, butynediol, is a defoliant, with effects similar to those of other defoliants.

Antiauxins are substances which compete biochemically with auxin for a site on an apoenzyme [Bonner & Bandurski (17)]; they might be expected to accelerate abscission, since auxin usually retards abscission. A few cases have been reported. TIBA (triiodobenzoic acid) and other halogen-substituted benzoic acids applied to the apical bud of a bean seedling induced abscission of the bud as well as deformation of the developing leaves [Weintraub *et al.* (130); Whiting & Murray (138)]. This abscission was prevented by simultaneous or previous application of IAA [Weintraub *et al.* (130)]. *Trans*-cinnamic acid accelerated abscission of debladed cotton petioles; the acceleration was reduced by simultaneous application of IAA [Hall (53)]. Several maleimides, shown to be antiauxins, accelerated the abscission of peach leaves [Overbeek *et al.* (97)].

The biochemical uses of enzyme inhibitors are described in a recent review [James (61)]. Since they interfere with the enzymatic functions required to keep an organ healthy they might be expected to accelerate abscission. The following have been so reported: iodoacetate [Carns & Addicott (24)]; phenyl mercurics [Brown & Addicott (18)]; and ethyl xanthate [Tharp (118)]. Several others are known to the reviewers to have been tested, but the results have not been published. As yet this information is too fragmentary to be of much help in understanding the physiology of abscission, but thorough

exploration of the field of enzyme inhibitors will provide information of great value.

Defoliants are chemicals which accelerate leaf abscission; they represent widely diverse chemical groups and include aminotriazole, calcium cyanamide, sodium acid cyanamide, calcium cyanate, butynediol, copper salts, sodium dichromate, sodium chlorate-borate, magnesium chlorate, sodium monochloroacetate, endothal (disodium 3,6-endoxohexahydrophthalate), and sodium ethyl xanthate [Hall *et al.* (54); Pridham & Hsu (101); Tharp (118)]. Knowledge of the precise physiological action of these substances is limited (see below). Their effects when applied are similar in injuring blades and petioles without seriously affecting abscission zones, stem, or buds. Properly applied defoliants usually do not interfere with the function of the abscission zone or with later growth from lateral buds. An exception is aminotriazole; it both induces leaf abscission and retards growth from lateral buds.

A question that has long troubled physiologists is whether the effects of abscission accelerants are direct (on abscission zone) or indirect (through their effects on leaf, fruit, etc.). It has been suggested that agents producing rapid acceleration probably act directly and agents producing relatively slow acceleration probably act indirectly [Kendall (63)]. The first part of this suggestion is reasonable, but the possibility that direct effects may be slow to appear cannot be excluded. Recent studies of defoliation give some evidence on this point. In cotton plants, removal of all but the lower 2 mm. of the leaf led to rapid abscission of the leaf stump, but a sodium chlorate-borate defoliant sprayed on the entire plant produced significantly more rapid abscission. Thus, the defoliant accelerated abscission to a greater degree than the removal of nearly the entire leaf [Addicott & Walhood (7)]. This result is not in opposition to the widespread belief that defoliants act indirectly through leaf injuries, but suggests that there are also direct effects on the abscission zone. In experiments with cotton explants, selected defoliants were applied individually, close to the abscission zone. No acceleration of the rate of abscission was observed. Since, in this material, abscission is initiated by excision, subsequent treatment can affect only the rate of abscission. This result suggested that the principal effect of defoliants (in the field) is to initiate abscission [Addicott *et al.* (5)].

Some of the leaf changes produced by defoliants are similar to preabscission changes in untreated leaves. Changes reported include the following: color changes [Hall & Lane (55); Johnson (62); Swets & Addicott (116)]; hydrolysis of carbohydrate and nitrogenous substances, and increased respiration [Hall *et al.* (54); Hall & Lane (55)]; disappearance of auxin [Swets & Addicott (116)]; and the production of ethylene [Jackson (59)]. The color changes and their patterns varied with defoliant and dosage. They included anthocyanin accumulation, chlorophyll loss, and browning or greying (from rapid desiccation with or without loss of chlorophyll). Sometimes only a portion of the blade was affected; injured areas were peripheral, central, or scattered. Some effects of defoliants can be at least partially prevented by

simultaneous application of auxin [Brown & Hyer (19); Hall (53)]. All changes produced by defoliants may not be of significance in abscission; since they are blade changes their abscission effects, if any, are indirect.

Fruit thinners include DNOC (dinitro-ortho-cresol), NAA, NAd (naphthaleneacetamide), MH (maleic hydrazide), IPC (isopropyl-N-phenyl carbamate) and 2,4-D [Batjer (12)]. Time for effective application varies from full bloom to four or five weeks after full bloom depending on the chemical. Since some of these chemicals are auxins it has been suggested that they accelerate abscission by accelerating growth in the abscission zone of the young fruit [Overbeek (95)]. The suggestion has not been supported by observations. Recently, Luckwill (78) found that NAA sprayed on open flowers before pollination induced incompatibility between pollen tubes and stylar tissue, accounting for thinning by sprays applied at this stage. NAA sprayed after petal fall led to the abortion of many developing seeds, accounting for thinning at this stage. In all his experiments Luckwill found no abscission for one to two weeks following NAA application, but after this period the thinning effect became manifest. Similar observations were made by Murneek & Teubner (90). DNOC sprayed on open apple flowers prevented germination of pollen, inhibited pollen tube growth, and killed the papillate cells of the stigma and the epidermal and outer carpellary cells of the style [Watson (129)]. Other fruit thinners may induce fruit abscission in the same way defoliants induce leaf abscission, since they appear to severely injure the fruit.

Hypotheses and Theories

Almost everyone who has studied abscission has speculated on the regulation of the process; few have resisted the temptation to put their ideas into print [Pfeiffer (99)]. Most speculations have treated a particular factor as the cause of abscission, overlooking or ignoring other factors. Only two theories, the hormone-ethylene balance theory [Gawadi & Avery (44)] and the auxin gradient theory [Addicott *et al.* (3)], have been supported by comprehensive arguments. The various hypotheses and theories known to the reviewers will be considered briefly.

Turgor.—Some of the early workers believed that abscission was brought about by increased turgor in cells of the abscission zone [Fitting (39); Goodspeed & Kendall (45)]. This idea apparently resulted from a misinterpretation of the common observation of turgid, often clavate cells on the freshly separated surfaces. The development of these cells is obviously a consequence of abscission under conditions favorable for cell enlargement. Under many conditions such cells are not observed. If present, those on the proximal abscission surface may mature, forming part of the primary protective layer. (For other effects of turgor see earlier subsection on *Water.*)

Organic acids.—In 1905 Wiesner (143) suggested that organic acids, produced in certain leaves during senescence, move to the abscission zone and there cause abscission by hydrolyzing cell wall constituents. Certain weak-

nesses in Wiesner's argument were pointed out in 1918 by Sampson (106). Facey (38) in 1950 concluded that her experiments supported Wiesner. The evidence presented by these three authors is summarized in the subsections on *Enzymes* and *Acidity*. It is probable that most preabscission changes have no relation to abscission. Wiesner's assumption that correlation is evidence of causal relation is of course untenable. The absence of direct measurements also makes untenable Facey's assumption that treatment of branches with hydrochloric acid or ammonia directly affects the pH of leaf abscission zones. Other interpretations of her results are more tenable, e.g., that the chemicals affected the auxin of the leaf blade. It is probable that changes in pH in the abscission zone accompany abscission, although the evidence is meager. The reviewers know of no measurements of pH since Sampson's work. It is hoped that considerably more information on the function of pH in abscission will soon be obtained.

Nutritional balance, competition.—It is widely recognized that the fruitfulness of many species is regulated by the relative abundance within the plant of carbohydrate and mineral nutrients (especially nitrogen) [Kraus & Kraybill (65)]. Since most species produce a superabundance of flowers, fruitfulness (i.e., number of fruits reaching maturity) depends in part on the extent to which flower and fruit abscission is prevented. Usually mineral nutrients are in ample supply, but carbohydrates limited; thus, June drop appears to follow unsuccessful competition for carbohydrates [Chandler (26)]. Carbohydrate and mineral nutrients may have some slight direct action on the abscission zone, but the probable sequence of events is as follows: insufficient nutrients lead to embryo abortion [Chandler (26)]; this drastically reduces the supply of auxin for the fruit abscission zone [Luckwill (77)]; and abscission results. This sequence is supported by the fact that, in some cases at least, application of auxin has either retarded or prevented young fruit abscission [Stewart & Klotz (115); Vyvyan (122); Walhood (126)].

Speculations on cotton fruit abscission.—Because of the economic importance of cotton and the fact that its fruit is easily shed, fruit abscission in cotton has been the subject of considerable study and speculation. Although the yield (fruit per plant) of cotton varies greatly with nutritional factors, the percentage of fruit abscised (per plant) is remarkably constant for each environment and variety [Eaton & Ergle (32)]. Only with extreme imbalance of carbohydrate and nitrogen is this relatively constant abscission percentage changed [Wadleigh (124); Mason (80)]. The subject has just been reviewed by Walhood (126). He found that fruit abscission ranges from about 30 to 70 per cent according to variety and environment.

The above generalizations appear to hold only when data for an entire season are summarized. Within a season there are important trends and often important transient effects. The cotton flowering period extends over about two months. At the start of the period fruit abscission is very low (circa 10 per cent), gradually increasing until at the end of the period, 90 to 100

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per cent of the young fruits are being abscised [Wadleigh (124)]. This makes it hazardous to compare fruit abscission at different stages of flowering. Transient effects may significantly change the fruit abscission percentage for a brief period. If the period is sufficiently brief this change is followed by a compensatory change leaving unchanged the seasonal average. Such effects have followed defruiting [Eaton & Ergle (32)], IAA application [Walhood (126)], brief insect injury, drought, flooding, clouds [Mason (80)], and excessive heat.

The nutritional balance theory obviously holds for yields in cotton [Eaton & Ergle (32); Wadleigh (124)], and appears to hold for fruit abscission under some conditions, as there is evidence that the cotton plant abscises young fruits which it cannot supply with adequate carbohydrate and nitrogen [Eaton & Ergle (32); Mason (80)]. However, in a number of cases the abscission percentage was high, although there was no evidence of an inadequate supply of nutrients [Eaton & Ergle (32)].

Eaton & Ergle's investigations (32, 33) have led them to postulate a relative fruitfulness hypothesis: for each variety and environment the number of fruits per unit fresh weight of leaves and stems is constant (reviewers' phrasing). Thus, within one experiment wide variations in yields may occur without affecting relative fruitfulness. Eaton has suggested that the regulating factors in fruit abscission are an antiauxin produced by older fruit and auxin produced by the leaves. These, then, interact in the fruiting branches, regulating the abscission of young fruit. Little is known of the auxin relations of cotton, but Eaton has cited unpublished investigations which, if extended, may support the auxin-antiauxin suggestion.

A simpler suggestion, which at present can be better supported, has been advanced by Wadleigh (124): a hormone from developing fruits stimulates the abscission of younger fruits. The requirements of this suggestion are completely met by auxin: auxin is produced by many developing fruits; the amount of auxin produced in young fruits is less than in older fruits [Luckwill (77)]; and nutrients are transported to and concentrated in regions relatively high in auxin [Emsweller & Stuart (35)]. Thus young fruits would receive fewer nutrients than older fruits, and, when the deficiency of nutrients in the young fruits was sufficient to reduce their auxin production, abscission would follow.

To the reviewers the nutritional balance theory adequately explains most of what is known of cotton fruit abscission. Nutrition appears to be interrelated with and affected by auxin, temperature, respiration, and translocation. These and still other factors combine to produce a percentage of fruit abscission relatively constant within any given variety and environment. These ideas are summarized in Figure 4.

Hormone-ethylene balance.—With a report of an investigation of leaf abscission in selected species, Gawadi & Avery (44) proposed a theory of the regulation of abscission which has been supported by Hall (53). Simply, the theory proposed that leaf abscission is regulated by the balance of hor-

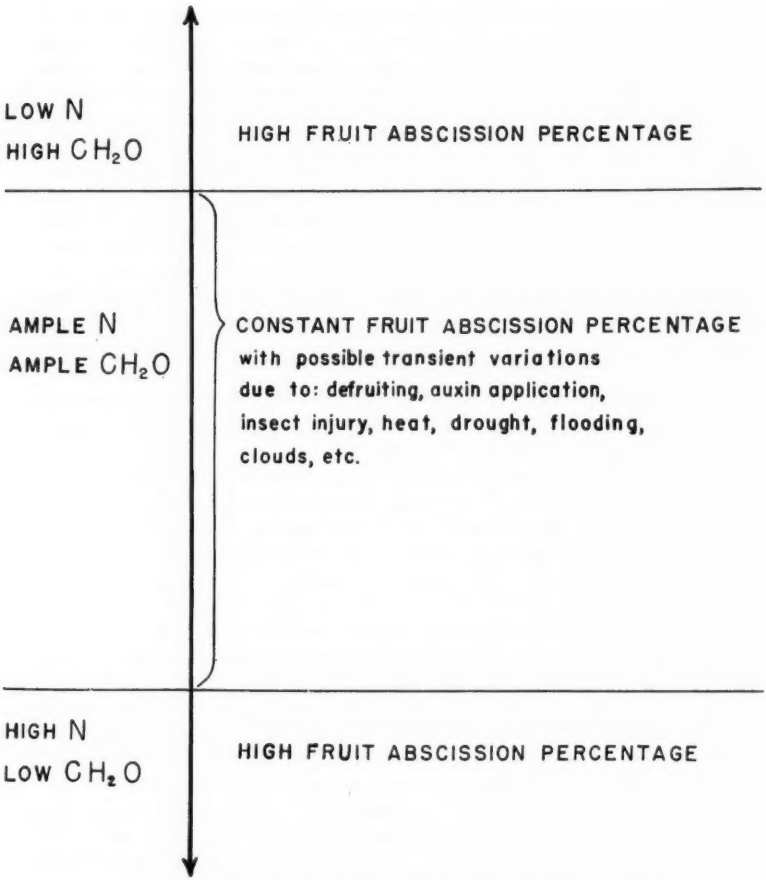

FIG. 4. Relations of factors known to affect fruit abscission in cotton.

mone (auxin) and ethylene in the leaf. In support of the theory Gawadi & Avery pointed out the following: leaf auxin diminishes with maturity [Avery (9); Goodwin (47)]; auxin retards the abscission of debladed petioles [LaRue (67)]; ethylene is produced by certain fruits [Gane (40)]; and ethylene accelerates ripening (aging) [Biale (15)]. It had been suggested that many plant parts, including leaves, produce ethylene [Denny (29); Denny & Miller (30)]. Gawadi & Avery assumed that ethylene was produced in leaves

and served to accelerate their aging and abscission. On this assumption and the above evidence they concluded that the interaction of the opposing factors, auxin and ethylene, regulated abscission. Hall (52, 53) found what he believed to be ethylene production in rose and cotton leaves and the IAA-inhibition of ethylene production in mold preparations. Working with debladed petioles of *Coleus* and cotton, Hall (53) did show that auxins reduced or prevented the acceleration of abscission by ethylene and ethylene chlorohydrin. In discussion he pointed out how the abscission effects of water, temperature, mineral nutrition, light intensity, and photoperiod could be interpreted as acting through intermediate effects on auxin or ethylene. This evidence and reasoning gave plausible support to the hormone-ethylene balance theory.

Since Hall's 1952 paper (53), considerable evidence has been presented which can be used to support the auxin part of the theory; little evidence has appeared to support the ethylene part. Indeed, recent evidence on ethylene not only fails to support the balance theory, but also raises serious doubts that endogenous ethylene has any regulating function. The leaf epinasty test is not specific for ethylene. The response is elicited by several substances including propylene, acetylene, butylene, and carbon monoxide. Denny & Miller (30) recognized this but, because the test was more sensitive to ethylene than to the other compounds, concluded that the emanations from plant parts were principally ethylene. In Denny's experiments (29) the plant material was usually cut into small pieces and the emanations collected over periods of several hours or days. These conditions would permit the development of microorganisms, a possible source of active emanations. Failure of microorganism development could account for the occasional failure to detect active emanations [Baile *et al.* (16)]. The production of active emanations by infected plants has been demonstrated [Williamson (144)]. Denny's experiments therefore do not afford a basis for concluding that leaves regularly produce ethylene.

The pea test, which has indicated that cotton leaves produce ethylene [Jackson (59)], is also nonspecific; it responds to ethanol as well as ethylene [Waggoner & Dimond (125)]. After years of study of the problem of ethylene identification, Biale *et al.* (16) recently concluded that an epinastic response in a closed system is insufficient for the identification of ethylene. Also unreliable are some chemical methods for the identification of ethylene. Hall's (53) method of identification of ethylene from rose and cotton leaves has been seriously questioned [Biale *et al.* (16)].

Only one identification of ethylene from leaves has been made with a universally approved method. Pratt (100) found that 26.6 kg. of fresh, green, detached, milk thistle leaves produced a total of 16.8 ml. of ethylene over a period of four days. In similar periods of time much less ethylene was obtained from leaves of tomato and chard.

The argument for ethylene production in leaves, by analogy with the well-

established fact of ethylene production in some ripening fruits is reasonable, but as outlined above there is little acceptable evidence that ethylene is produced by healthy leaves. Moreover, ethylene is highly volatile and, if produced by leaves in the open, should rapidly escape. It is doubtful that it could be transported from the leaf blade to the abscission zone in sufficient quantity to affect abscission. In field experiments, no leaf abscission was found after release of ethylene or after spraying with solutions containing as much as 300 per cent ethylene [Addicott *et al.* (6)]. Further, 20,550 liters of the air in a cotton field during a period of normal leaf abscission were passed through a trapping agent and tested for ethylene. A concentration of 1.7 parts per billion was found. This was at the limit of sensitivity of the test and was considered to be inconclusive evidence of ethylene production by field cotton [Addicott *et al.* (6)].

The greater part of our limited information on the physiology of ethylene has come from studies of fruit ripening. Pertinent to the present considerations are the following facts: some fruits (e.g., lemon, orange, mango) do not produce ethylene as they ripen; and in fruits producing ethylene, its production either coincides with or follows the ripening (climacteric) rise in respiration, depending on the species. Biale *et al.* (16) concluded "ethylene is a product of the ripening process rather than a causal agent."

Thus, although ethylene may be produced by some leaves under some conditions it must be tentatively concluded that ethylene is probably not an endogenous regulator of leaf abscission. Rather, ethylene appears to be an interesting type of abscission accelerant, which happens to be produced by some fruits during or following ripening.

Auxin gradient.—Many of the recent advances in auxin physiology have a bearing on abscission and indicate that auxin is an important factor in abscission. Abscission physiology shows that auxin has a dominant role "in controlling the normal order and intervals of leaf abscission" [Wetmore & Jacobs (137)], and is "the principal endogeneous regulator of abscission" [Addicott *et al.* (3)].

Measurement and comparison of auxin concentrations distal and proximal to the abscission zone suggested that the auxin gradient across the abscission zone was more important in the regulation of abscission than the auxin concentration in the abscising organ [Shoji *et al.* (110)]. Acceleration of abscission by proximally applied auxin supported this suggestion [Addicott & Lynch (2); Swets & Addicott (116)]. Consideration of all available information showed that a satisfactory explanation can be provided by an auxin gradient theory [Addicott *et al.* (3)].

This theory proposes that onset and rate of abscission are regulated by the auxin gradient across the abscission zone: abscission does not occur when the gradient is steep, that is, high on the distal side and low on the proximal side of the abscission zone. Abscission occurs when the gradient becomes gentle or disappears and is accelerated when the gradient is reversed. Figure 5

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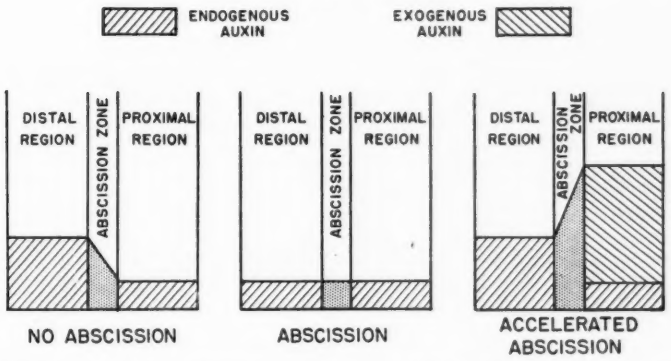

FIG. 5. Relations between the auxin gradient across the abscission zone and abscission.

diagrams these relations. There is evidence of positive correlation between the rate of change of gradient and rate of abscission.

The theory is supported by (*a*) the lowering of the auxin gradient across the abscission zone previous to abscission and (*b*) the acceleration of abscission by proximal application of auxin (see subsection on *Auxin*). The accelerating effect of neighboring young leaves on the abscission of debladed petioles [Rossetter & Jacobs (105)] is attributed to auxin produced by the young leaves [Jacobs (60)]. The effects of various internal and environmental factors on abscission parallel their effects on auxin, predominantly on the concentration distal to the abscission zone. The stem, the principal organ proximal to abscission zones, is characteristically low in auxin [Went & Thimann (134)].

Factors not yet known to affect auxin can be logically conceived to affect it. Further, the theory provides explanation for the perplexing observation that applied auxins may fail to retard abscission and may even accelerate it (see subsection on *Auxin*). For example, applied auxin could accelerate abscission by lowering the effective distal concentration through competitive interaction with the endogenous auxin, or, if transported, by raising the proximal concentration. The possible function of the auxin gradient in the dehiscent type of abscission has not been considered. No pertinent physiological information is known.

It should be made clear that the auxin gradient theory is not intended to imply that other factors are not important in the regulation of abscission. The theory proposes only that auxin is the principal regulator—the factor most often limiting abscission. Obviously, other factors at times limit or regulate abscission. Such factors include nutrient relations, water relations, temperature, and oxygen as discussed earlier.

TABLE II

SUMMARY OF THE USES OF ABSCISSION REGULATORS*

Organ abscissing	Plants	Accelerants†	Ref.	Retardants†	Ref.
Stem	*Mirabilis*			IAA	(13)
Stem bud	Bean	TIBA, etc. MH	(130) (48)		
Bark	Aspen	Sodium mono-chloroacetate 2,4-D 2,4,5-T	(98)		
Leaves	Apple			NAA 2,4-D 2,4,5-T 2,4,5-TP	(34)
	Bean	Ammonium thiocyanate Endothal Sodium cyanamide TIBA	(116) (82) (138)		
	Cabbage Cauliflower			NAA 2,4-D	(69)
	Cherry			NAA 2,4-D	(121)
	Cotton	Aminotriazole Butynediol Chlorates Cyanamides Dichromates Endothal Ethyl xanthates	(54) (50) (118)	 IAA NAA	(53)

* Both experimental uses and established practices are included. Most retardants are derivatives of organic acids, such as salts, esters, amides and amines. The derivatives are not named. Effectiveness depends on derivative used and conditions of application.

† List of abbreviations [Norman (93)]:

ClPA	*para*-Chlorophenoxyacetic acid
ClPP	alpha-*ortho*-Chlorophenoxypropionic acid
DNOC	2,4-Dinitro-*ortho*-cresol
IAA	beta-Indoleacetic acid
IBA	beta-Indolebutyric acid
IPC	O-Isopropyl-N-phenyl carbamate
MCPA	2-Methyl-4-chlorophenoxyacetic acid
MH	1,2-Dihydro-3,6-pyridazine-dione (Maleic Hydrazide)
NAA	alpha-Naphthaleneacetic acid
NOA	beta-Naphthoxyacetic acid
TIBA	2,4,5-Triiodobenzoic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4,5-TP	2,4,5-Trichlorophenoxypropionic acid

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TABLE II—(*continued*)

Organ abscissing	Plants	Accelerants†	Ref.	Retardants‡	Ref.
	Holly	Ethylene	(84)	NAA	(84)
	Nursery stock	Endothal Copper salts	(101)		
Inflorescence	Olive			2,4-D	(56)
Flowers	*Begonia*			NAA	(128)
	Dogwood			ClPA NAA	(135)
	Flowering cherry			IAA NAA NOA	(135)
	Lima bean			ClPA+IBA	(136)
	Snap bean			ClPA ClPP NAA NOA	(145)
	Lupinus			IAA NAA	(127)
Style	*Lilium*			NAA	(35)
	Cherry *Oenothera* Plum			NAA	(70)
	Petunia			NAA	(37)
Calyx (fruit button)	*Citrus*			2,4-D 2,4,5-T	(113)
Young fruit	Apple	DNOC NAA	(12)	NAA	(123)
	Cherry‡			NAA 2,4-D	(148)
	Olive	NAA	(57)		
	Peach	DNOC IPC MH NAA	(12) (79)		
	Catalpa Eastern poplar European ash Honey locust Horse chestnut	NAA	(86)		

‡ The literature is not clear as to whether the increased fruit set is attributed to reduction of abscission, stimulation of fruit development, or both.

TABLE II—(*continued*)

Organ abscissing	Plants	Accelerants†	Ref.	Retardants†	Ref.
	Ginkgo	MH	(86)		
	Bean			ClPA+IBA	(136)
	Cantaloupe‡			IAA	(20)
	Tomato‡			ClPA IAA IBA NAA NOA 2,4-D 2,4,5-T etc.	(8) (145)
Mature fruits	Almond			2,4-D 2,4,5-T	(109)
	Apple			MCPA 2,4-D 2,4,5-T 2,4,5-TP NAA	(112) (12) (8)
	Apricot Peach			NAA	(12)
	Cherry			NAA	(146)
	Citrus			2,4-D 2,4,5-T	(114)
	Pear			NAA 2,4-D 2,4,5-T	(12)
	Holly			NAA	(84)
Fruit hull	Walnut	Ethylene	(112)		

Agricultural Regulation of Abscission

The regulation of abscission has become an agricultural practice of considerable importance. Recent publications include various aspects of the use of abscission regulators [Audus (8); Tharp (118); Tukey (120)], but none include a full summary. Table II is intended to include known uses, experimental and practical. Key or typical references, or both, are cited but no attempt was made to cover the voluminous horticultural and agronomic literature which can be found in appropriate journals. Many chemicals known to have little or no effect on abscission are not included. Gardner & Cooper (41) list 156 chemicals with little or no ability to retard abscission of debladed petioles and Weintraub *et al.* (130), from their experiments with chemicals

inducing stem bud abscission, list 55 chemicals with relatively low activity and 519 with no activity. In addition, thousands of chemicals have been tested by chemical manufacturers but, unfortunately, the results are rarely published.

General Considerations

Some observations not directly related to abscission provide evidence which may be helpful in understanding it. For example, there are striking similarities between the growth of lateral buds [Thimann (119)] and abscission: the apical bud inhibits the growth of lateral buds, the leaf inhibits its abscission; growth of lateral buds is initiated by removal of the apical bud, leaf abscission is initiated by removal of the leaf blade; and the growth of lateral buds and abscission are both inhibited by auxin. A comparative study of growth of lateral buds and abscission should provide valuable information about both. Studies of fruit ripening should also provide information of value to the study of abscission. Both processes involve complicated physiological changes, especially in cell wall constituents. Moreover, knowledge of fruit ripening is now more extensive than knowledge of abscission, and fruit materials can be obtained in much greater volume than abscission zone materials.

In conclusion it should be pointed out that although our knowledge of abscission is broad in scope, it is not deep. Numerous problems are yet to be solved, including the following: the factors underlying the widely differing percentages of abscission in related varieties of plants; the relation between auxin and cytolysis; the factors confining cytolytic changes to abscission zones; the mode of action of ethylene, oxygen, and other abscission accelerants; and the identification of enzymes active in abscission. These and many other problems must be solved before we can materially advance our understanding of the physiology of abscission.

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THE WATER ECONOMY AND THE HYDRATURE OF PLANTS[1]

By H. Walter

Department of Botany, State College of Agriculture, Stuttgart-Hohenheim, Germany

Introduction

This treatment of the water economy of plants differs from that usual in physiology. Typically, the water relations of plant cells and tissues are discussed first, followed by consideration of water absorption by roots, transpiration of the leaves, water conduction, etc., each part treated separately. In doing so it is frequently forgotten that the plant as such is a unit and that these parts of water economy are interrelated and dependent on each other. They can scarcely be separated in a plant growing under natural conditions. The attempt shall therefore be made to understand the water economy of a plant as a whole, and to discuss the variations shown by the separate groups of plants distinguished by the complexity of their organisation.

The importance of water economy appears in the fact that most of the weight of the fresh plant, particularly the protoplast, is represented by water. A proper understanding of the water economy of plants is only achieved, however, if it is taken into consideration that not only is the quantity of the water important but, above all, the "condition of the water" in the living substance; its "water potential."

We may demonstrate this condition by the use of four examples: (*a*) Let us assume that in one cubic meter of air there are 12 gm. of water vapour, and that the temperature in one case is 15°C. and in another case 20°C., respectively. Supposing that in each case a piece of bread is introduced into this air. Mould will grow on it at 15°C. but not at a temperature of 20°C. A temperature of 20°C. does not prevent the growth of mould, and the same amount of water is at the disposal of the mould in either case, but the "condition of the water," as shown by the relative humidity, differs. The latter at 15°C. is 94 per cent but at 20°C. only 70 per cent.

(*b*) Bean seedlings are to be transferred into Knop's culture solution, each of the containers have a capacity of 1 l. of water. In one container the culture solution is left unchanged. In the other, 171 gm. of saccharose are added, so that the osmotic pressure is increased considerably. Though the quantity of water at the disposal of both sets of plants is the same, those in the normal culture fluid will grow well, while those in the fluid with added sugar will wilt or perish.

(*c*) To two vessels containing 10 kg. of sandy soil and of clay soil, respectively, a measured quantity of water is added regularly so that the soil

[1] The survey of the literature pertaining to this review was completed in August, 1954.

always contains 10 per cent water. Oats are then planted in the soils. They will grow in the sandy soil but not in the clay. In this case, too, the same amount of water is at the disposal of the plants, but the "suction tension" of the water differs.

(*d*) For a last example, 100 gm. of millet seeds and pea seeds, respectively, may serve. These are allowed to soak in water until each lot has absorbed the same quantity, the amount being 30 gm. for each. The millet seeds with this intake reach their swelling maximum and will germinate. The pea seeds have a maximal swelling capacity of 100 gm., and therefore will not germinate at a water content of 30 per cent.

These four examples demonstrate that the quantity of the water alone is not a sufficient characteristic of the water economy of plants. The differences in behavior are explained by differences in relative humidity, in osmotic forces, in suction tension of the soil, or by variations in the degree of swelling of seeds. All these phenomena, however, may be reduced to one factor, the "water condition," which is measured by the relative water vapour pressure.

For this "water condition" the expression "hydrature" was introduced by the author as a result of his research at the Desert Laboratory in Tucson, Arizona (1, 2). The hydrature $(Hy)^2$ is 100 per cent when the relative water vapour pressure is equal to that of chemically pure water. It is less than 100 per cent when the relative vapour pressure is less than that of chemically pure water. In the examples given, the lower the hydrature the more the growth of a plant is retarded. What we learn from the first example is that mould may grow at 94 per cent but not at 70 per cent of hydrature. In the second example, too, the relative vapour pressure, and therefore the hydrature, of the culture solution with sugar is lower than that of the normal culture solution. In the latter it is scarcely under 100 per cent, but upon the addition of sugar it will be lower than 99 per cent. The same applies to the third and fourth examples. The hydrature of sandy soil with a water content of 10 per cent and that of millet seeds with a maximal water content is 100 per cent, while that of clay soil with a water content of 10 per cent and that of the pea seeds which have not reached their swelling maximum is less than 100 per cent.

In terms of hydrature, it appears that two fundamentally different types of plants may be distinguished: (*a*) poikilohydrous plants in which the hydrature is completely dependent upon the surrounding; and (*b*) homoiohydrous plants in which the hydrature is stabilized in a large measure, independently of the surroundings. Both groups will be discussed in more detail.

Poikilohydrous Plants

Members of this group are the lower plants such as bacteria, algae, fungi, lichens, and mosses. They live in a medium which is constantly of a very high hydrature (fresh water, dilute culture solution). When the hydrature of the surroundings is decreased (concentrated culture solution, air which is

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not saturated with water vapour) these plants eventually stop growing. When the hydrature of the surroundings is low, the plants lose their water until their hydrature is adjusted to that of the surrounding medium, i.e., they dry out and pass into a state of latent life. This applies to those lower plants also, which, like the algae of the air, e.g., *Pleurococcus* or *Trentepolia*, lichens, and bryophytes, are adapted to life in the air.

For practical purposes it is extremely important to know at which hydrature bacteria or moulds, for example, are able to grow. To this end usually the critical "water content" has been established, as with hay, corn, flour, wood, and so on. It appears, however, that not the water content but the "hydrature" only of the involved substrate is of importance. There is an absolute lower limit of hydrature, assuring a certain preservation of all substances by dehydration. For practical purposes the limit is established at 75 per cent Hy. Although Snow (3) was able to demonstrate that even at a relative vapour pressure of 66 per cent, or even 62 per cent, exceptional germination of spores of moulds took place after two years, this is of no importance for the preservation by dehydration. Even at 50 per cent relative humidity a single cell division of *Pleurococcus* is mentioned (4, 5) but proper development of the alga appears possible only at a very high hydrature.

To learn the relationship between the conditions of hydrature and the development of an organism is of utmost importance. It is found that a decrease in hydrature at first causes only retarding of growth; at a still lower hydrature, for practical purposes, growth stops completely. Burcik (6) has done research into these conditions with bacteria and yeast. Some examples of the lower limits of hydrature will be given here. With certain species different cultures may show certain variations. The figures mean those percentages of the relative vapour pressure, i.e., of hydrature, at which growth stops; the figures in parentheses indicate the start of decreasing growth: *Bacillus mycoides*, 99; *Pseudomonas pyocyanea*, *Bacillus luteus* and *Bacillus asterosporus*, 98; *Bacillus radicicola* (99) 98; *Pseudomonas tumefacines* (99) 96; *Bacillus mesentericus* (98) 95.5; *Bacterium coli* (98) 96 (other strain 93.5); *Bacillus subtilis* (98) 95; *Bacterium prodigiosum* and *Bacterium aerogenes* (98) 94.5; *Micrococcus roseus* (95.5) 90; *Saccharomyces cerevisiae* (97.5) 89; *Endomyces vernalis* (95) 88.

These examples show that, in general, bacteria need a rather high hydrature for growth, while yeasts, being much less sensitive, are able to flourish in solutions of high concentrations. They are surpassed even in this respect by certain moulds (7, 8), although the following distinctions must be made: (*a*) hygrophilic forms in which each decrease in hydrature leads to a slowing of growth which stops completely at 97 to 92 per cent Hy, e.g., *Merulius lacrimans* or *Oidium lactis;* (*b*) mesophilic forms in which growth first slows down at 97 to 95 per cent Hy and stops at about 88 per cent Hy, e.g., *Mucor* and *Rhizopus nigricans;* (*c*) xerophilic or, better, xerotolerant species showing no retardation of growth to 90 per cent Hy, and growth of which is possible

to the absolute minimum of hydrature, that is, to 70 per cent Hy, e.g., *Penicillium* and *Aspergillus*. These moulds therefore spread farthest and are the most frequently found.

Closer examination shows the minimum hydrature to be not absolutely constant but to become lower, the more favourable the other growth factors are, for instance, the temperature. The minimum hydrature for *Aspergillus glaucus* is kown to be 77.5 per cent Hy at 12°C., 70 per cent Hy at 30°C., but 85 per cent Hy at 40°C. For practical purposes, it is important to know that wood-destroying fungi may grow at 85 per cent Hy, though damage worth mentioning is produced only above 95 per cent Hy (9).

For phytopathologists it is of interest to know the limiting values for the germination of the spores and conidia of the various pathogenic fungi. Examples which may be mentioned are: *Cercospora beticola* 97 per cent Hy, *Sphaerotheca pannosa* 95 per cent Hy, *Alternaria solani* 92 per cent Hy, *Alternaria citri* 84 per cent Hy (10). Similar to the behaviour of the spores of fungi or of conidia is that of the pollen grains of flowering plants. Usually they develop only into a pollen tube if the hydrature is more than 98 per cent. The lowest value found to date is 95.9 per cent.

Not only may the hydrature of a medium in which organisms are developing be decreased by less moisture, but also by osmotically-active substances if the organisms are grown in solution. In general, it has been shown that it does not matter to the organism whether it grows on a solid substratum in moist air or in solution, as long as the osmotically-active substances have no special effects Usually, the limiting values of hydrature tend to be rather lower on cultivation in solutions. Those organisms which are able to live in salt water or even need salt for their development may be an exception, however. The organisms concerned are halophilic and their physiology is a separate problem. We shall return to this point in the last section of the review.

Homoiohydrous Plants

In contrast to poikilohydrous plants whose hydrature is completely determined by that of their environment, homoiohydrous plants have a hydrature which is largely independent of that of their environment. The spermatophytes or angiosperms may be considered as types of homoiohydrous plants. They insert themselves into the gradient of hydrature which exists between the soil and the atmosphere. Their roots spread in the moist layers of the soil, of which the hydrature is usually more than 99 per cent, while the shoot and the leaves rise into the lower layers of the atmosphere of which the hydrature may be 60 per cent on sunny days, and in desert country may even sink under 20 per cent. The higher plant is largely insulated from its surroundings by epidermis or cork tissue. However, this insulation is never complete and the plant makes use of the gradient of the hydrature in which it exists to create, without expending its own energy, a current of water from its roots to its leaves. In doing so, water intake and expenditure is regulated so that the hydrature of the living organs varies only within certain limiting values.

These values will always be rather less than the hydrature of the soil layer but always more than the average hydrature of the surrounding air.

Only in extreme cases are there exceptions to this rule. The succulents of the desert, for instance, may root in strata which are absolutely dry during most of the year. In this case there is no water intake and the plant lives on the reserves of water collected during the wet season. On the other hand, in the tropical primeval forest the air may be saturated with water vapour for days, the hydrature being 100 per cent. In such case, there will be no transpiration current and the plant must spend energy in guttation, i.e., in excreting drops of water. We shall, however, set aside these extreme cases.

"Hydrature" of homoiohydrous plants shall be understood to be the hydrature of the protoplast of the cell. It is known that the degree of swelling of live plasm is dependent upon the relative vapour pressure, i.e., the hydrature, in the same way as that of dead hygrophilic colloids. The protoplast of the carpospores of *Lemanea*, for, instance, behaves in this respect like a mixture of equal parts of gelatine, casein, and nuclein (2). Each decrease in hydrature causes dehydration of plasm. There is an equilibrium between the hydration of the plasm and the concentration of the cell sap, the latter being determined by its osmotic value. Therefore, the concentration of the cell sap in atmospheres, i.e., the osmotic value, may serve as a measure of the hydrature of plasm.

It must be observed, that the terms lately used in American research for osmotic values differ from those in Europe. To avoid errors, the terms used to describe the conditions of the cell are given in both forms.

In America:[2] Diffusion pressure deficit (DPD) = osmotic pressure (OP) − turgor pressure (TP).

In Europe:[2] Suction tension (S) = osmotic value (W) − turgor pressure (P).

It does not appear to be useful to consider a measurable physical quantity (suction tension) as a difference between two nonmeasurable quantities (diffusion pressures). Moreover, in physics, a factor is not usually expressed by two (OP, TP) or even three letters (DPD). It is also preferable to speak of "osmotic value" rather than "osmotic pressure," since the latter is not a measurable pressure in contrast to "turgor pressure," the turgor pressure being a hydrostatic pressure. These questions of terms for osmotic values have been variously discussed lately (11 to 21), with no agreement about them having been reached. Therefore, the European terms which are thought to be correct, will be used in this paper.

Suction tension is important because it controls the movement and the distribution of water among the various tissues. For the hydrature of plasm the osmotic values are decisive since the imbibitional forces of the protoplast are always in equilibrium with the osmotic forces of the vacuole solution.

[2] The following abbreviations will be used: Hy, hydrature; DPD, diffusion pressure deficit; OP, osmotic pressure; TP, turgor pressure; S, suction tension; W, osmotic value; P, turgor pressure.

The hydrature of the cell sap and therefore of the plasm in higher plants usually is between 100 per cent and 98 per cent, corresponding to osmotic values between 0 and 27 atm. In extreme cases only are the values higher; in very rare ones even over 100 atm. Since the variations in hydrature are, in general, extremely small it is more convenient not to express the hydrature of the protoplast in per cent Hy, but by the osmotic value of the cell sap in atm. An increase in osmotic value corresponds to a decrease in hydrature.

The hydrature is not the same in all parts of a homoiohydrous plant. Usually the osmotic values increase from the roots to the tips of the shoot. Young organs have lower osmotic values than mature or old ones; leaves grown in the sun have higher ones than those grown in the shade. Flowers, fruits, and storage organs deviate in either direction. Comparable organs of the same species under optimal conditions show a definite value, however. Therefore, the hydrature or the optimal osmotic value is a specific character. A comparison of the same alpine species of the Rocky Mountains and the Alps, for instance, shows the same osmotic values in either (41). Each disturbance of the water economy of a plant immediately causes an increase in the osmotic values, regardless of whether the disturbance is caused by increased water expenditure, by decreased water intake through drying of the soil, or by both factors together. The osmotic value may serve as an extremely exact indicator of the water balance and, therefore, of the total water economy of a plant (2).

Before discussing these more physiological questions, it may be observed that there are intermediate forms connecting typically poikilohydrous lower plants and typically homoiohydrous higher plants. Dehydration at decreasing hydrature of the surroundings may be slowed down by a gelatinous coating of the cells as demonstrated by *Nostoc*. The cortical hyphae of the fungi in lichens, being capable of a high degree of hydration, retain water for a time and so lengthen the duration of favourable conditions of hydrature for photosynthesis by the algal component. Many mosses are capable of storing water in their cushions by capillary forces, especially species of *Sphagnum* and *Leucobryum*.

In some fungi, however, especially hymenomycetes which produce large fruiting bodies, a change to homoiohydrous habits may be observed. These rise above the surface of the soil, have a certain amount of transpiration and are provided with water from the mycelium. With *Merulius*, vascular hyphae are known to be developed in its branches, making it possible for this destructive fungus to attack dry wood while attached to moist wood. Homoiohydrous habits, to a degree, were also observed in bryophytes, for instance, *Marchantia* (22), which through rhizoids is able to extract water from the soil. A kind of water current is also produced in *Polytrichum*.

Prothallia of pteridophytes are of a decidedly poikilohydrous type. Only the development of a root system and conductive tissues proper allowed the sporophytes of the pteridophytes to develop homoiohydrous habits. Most ferns (Filicineae), horsetails (Equisetineae), and clubmosses (Lycopodineae)

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may be called homoiohydrous plants. Ferns, as far as they occur in dry habitats or in arid regions, retain the faculty of drying completely like poikilohydrous plants when the hydrature of their surroundings is low. They then revert to a state of latent life reviving under favourable conditions of hydrature (after rain) only.

In temperate regions ferns which, like *Polypodium vulgare* and *Asplenium ruta muraria*, live on rock or in crevices of rock may, for a short time, survive almost complete dehydration. Definitely poikilohydrous species are the more continental *Notholaena maranthae* and *Ceterach officinarum* (26); the same applies to the species of the genera *Ceterach*, *Notholaena*, *Pellea*, *Cheilanthes*, *Actiniopteris*, *Selaginella* and others which occur in the arid regions of South Africa and America (1, 23). When drying because of cohesion tension, their leaves roll in (24). This type no longer occurs among gymnosperms, although exceptional species of *Pinus* and *Juniperus* intrude far into arid regions, while *Ephedra* as well as *Welwitschia* are part of the desert vegetation. Strangely enough, among angiosperms there are some types which may be completely dehydrated for many months without harm, while in foliage. Therefore, they, too, must be considered poikilohydrous species. Examples are *Carex physodes* of Central Asia, the dwarf shrub *Myrothamnus flabellifolia* of South Africa as well as species of *Barbacenia* (25). More ambiguous is the behaviour of *Craterostigma* of South Africa and of Hiberian-Balcanic gesneriaceae *Ramondia* and *Haberlea* (1).

Iljin (27, 28) declares that all plasm, even that of cells of higher plants, is able to withstand complete dehydration when it is done so that mechanical damage is avoided. This opinion appears to be lacking a satisfactory experimental foundation, although it must be conceded that the faculty may be present potentially. The cells of seed embryos adopt the hydrature of the environment like poikilohydrous plants.

The "Bound Water" Theory

Hydration curves of various hygrophilic colloids as a function of the relative water vapour pressure always are S-shaped (29, 30). The curve rises slowly at first then very steeply, and at about 96 per cent Hy assumes an almost horizontal direction. Therefore, a colloid of maximal hydration will lose, at the outset, a relatively large amount of water when the hydrature is decreased slowly and evenly; thereafter, the water loss gradually diminishes. The curve is continuous and without angles, however, indicating that there is no difference between "free" and "bound" water. As soon as the hydrature sinks below 100 per cent the water is "bound" to a degree; the lower the hydrature, the more strongly it is "bound." Any sharp distinction between "free" and "bound" water is to be considered arbitrary. The water may be assumed to be completely bound at a hydrature of 96 per cent, the binding force then being 55 atm., but such a proceeding is without logical foundation and this might as well be done at 98.5 per cent (=20 atm.) or at any other value. Weismann (31) has theoretically and experimentally

discussed the "bound water" theory. He concluded that by application of the various methods used to determine the quantity of bound water no fundamental difference between true solutions and colloid solutions is found. There is no reason, therefore, to distinguish bound and free water in colloids in contrast to solutions. By the calorimetric, dilatometric, and cryoscopic method of Briggs (32) values are obtained which are expressed by the relation of relative vapour pressure, that is, hydrature, to water content. By Newton's (33) and Gortner's (34) cryoscopic methods, Hill's (35) vapour pressure method, Levitt's & Scarth's (36) plasmolytical method, and Lebedincew's (37) dilatometric method only the deviations from the laws of an ideal dilute solution are determined, and these in arbitrary and differing ways. The presence of a particular bound water is no more indicated by Mason's & Phillis' (38) methods, Greenberg's & Greenberg's ultrafiltration method (39), and Newton's pressing out method. Therefore, it would be better to drop completely the conception of "bound water," and to give, instead, the respective degree of binding of the water by the hydrature or the corresponding osmotic value.

In error also, is the assumption that the lower freezing point of living tissue means a larger amount of bound water. Only an inaccurate determination of the freezing point is the reason for such a conclusion, since the continuous stirring of the solution that is required is not possible. Here, determination of the heat liberated during freezing establishes that the osmotic value of the cell sap of living cells is exactly equal to that obtained from pressed-out cell sap after killing the cells (40). The crysocopic method of determining the osmotic value of cell sap has proved most reliable when handled with the necessary care, although only averages for the respective tissues or organs are obtained.

The Physiological Importance of Hydrature in Higher Plants

Every difficulty in the water economy of a plant leads to an increase of the osmotic value of the cell sap and, therefore, to a decrease of the hydrature of the plasm. The latter leads, everything else being constant, to a certain dehydration of the plasm and therefore to changes in the activities of life. If the supply of water by the roots is constant, difficulties in water economy may be caused by permanently increased water expenditure, as, for instance, that of leaves exposed to the sun compared to leaves protected by the shade. In agreement with this is the fact that the osmotic value of leaves exposed to the sun, even when fully turgid, is always higher than that of leaves of the same plant which are shaded. Similar circumstances result where water intake is made more difficult as in dryer soils. When plants are cultivated in soils having a water content of 70 per cent and 30 per cent of their capacity respectively, the osmotic value will be higher in the latter soil. If a species grows both in dry and moist environments, very small differences in humidity are indicated by increased osmotic values. Where wet

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and dry seasons alternate during the vegetative period, differences in osmotic values may also vary successively. The osmotic value then shows a definite yearly periodicity, reaching a maximum during the dry season (41). How strongly the various species react to such differences depends upon their specific constitution. Only among individuals of the same species may the absolute osmotic values be compared. This rule has appeared again and again, so that special examples are not required (41). It shall be mentioned only that the behaviour of hydrophytes appears to differ.

It may also be considered a general rule that an increase of the osmotic value beyond the optimal leads to a reduction of the activities of life, e.g., of growth, photosynthesis, and respiration. This is true of the latter only if no injuries are experienced. Here, too, the reactions of different species vary extremely. With some a slight decrease in hydrature leads to a strong reduction in activity, while with others photosynthesis may continue in spite of a large increase in the osmotic values, as Eckardt demonstrated for a Mediterranean species of *Centaurea* (42), and Oppenheimer for *Phillyrea media* (43). Growth is not only reduced in quantity, but there is also a qualitative change, so that the newly formed organs are of a different morphological structure. The leaves of *Encelia farinosa*, a plant characteristic of the Sonorian desert, when formed under favourable conditions of water supply (that is, under a high hydrature) are completely different from those grown under conditions produced by a poor water supply (when hydrature is low) (1). In a less striking way this applies to practically every species (41). It is not, therefore, surprising that characteristic limiting osmotic values are found for the various ecological types of the diverse climatic regions imposed by the changing conditions of water supply. Extremely characteristic osmotic spectra may be worked out for each region by considering the species which occur there, as has been done for Arizona by the author (900 values). The following ecological types were distinguished: cacti, leafy succulents, winter ephemerals, summer ephemerals, raingreen species (*Fouquieria*, *Jatropha*, *Elaphrium*), deciduous trees and shrubs, poikilohydrous ferns, mesomorphic shrubs, xeromorphic shrubs, and perennial malakophyllic species. Similar spectra exist for the prairie (600 values), for the Rocky Mountains (450 values), and for certain regions of Europe, Asia, and Africa (41).

The connection between the conditions of hydrature of cultivated plants and the quantity of the yield is very distinct. Lobov (44) was able to demonstrate that a definite reduction of growth is produced in young tomato plants by an increase of the osmotic value to 10 atm. While the values at night were between 6.7 and 7.3 atm., they rose, during the day, to 10.2 atm. Concomitantly, at night the increase in height of the plant was 1.84 cm. as compared to 0.45 cm. during the day. Therefore, when using artificial irrigation it is necessary to avoid an increase in the concentration of the cell sap. Experiments were done with several species of vegetables which were

watered: (*a*) when the osmotic value rose to 8 atm.; (*b*) when it reached 10 atm.; (*c*) when the increase had reached 12 atm. The yield in hundreds of kg. per hectar are listed in Table I.

Stieglitz (45) compared the osmotic values and the yield of winter wheat in a normal and in a dry year. In a normal year the osmotic values varied from 13 to 17 atm., from April to August. In the dry year from June to August they rose from 18 to 25 atm. Accordingly, the yields were, with full manuring, in the normal year 45 kg. per are (100 square meters) of grain and 105 kg. per are of straw but 22 kg. per are of grain and 28 kg. per are of straw in the dry year. Observe how the yield of grain is decreased by 50

TABLE I

EFFECT OF TIME OF WATERING ON YIELD

	Plant Osmotic Value at Time of Watering		
	8 atm.	10 atm.	12 atm.
	Yield in Hundreds of Kg. per Hectar		
Early cabbage	463	418	217
Early potatoes	217	183	152
Early tomatoes	530	540	420

per cent and that of straw by 75 per cent by the adverse conditions of hydrature.

It must be stressed that the hydrature of the plasm is only one of the factors upon which the growth and the development of plants depend. When water is the limiting factor for plant life, that is, in years of drought, or, always, in arid regions, the conditions of hydrature will appear to be of special influence. The problems of xerophytes, for instance, will be understood only when the conditions of hydrature are considered. In a humid climate other factors may be of more importance still. This is especially true when the effects of hydrature on the plasm appear jointly with those of electrolytes.

THE ACTION OF ELECTROLYTES AND THE PROBLEMS OF HALOPHYTES

So far, only a change in hydration caused by hydrature has been considered. It is known, however, that the hydration of proteins, and therefore of plasm, is largely dependent upon pH values or the influence of electrolytes, cations as well as anions. In addition to hydration proper which consists only of water intake or loss of water on change in hydrature without change in the plasm, there exists another hydration, that by electolytes. In the latter certain electrostatic or physico-chemical changes of the plasm are produced. These changes in hydration are examined physiologically in

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the laboratory, mainly, often using ions and concentrations which the plant would never meet under natural conditions, although a certain quantity of electolytes is contained in the cell sap of all plants. Knodel (46) has examined the chemical components which produce the osmotic values of the cell sap of some leaves. It was found that only in evergreen leaves (*Picea, Buxus*) is the sugar concentration in atm. higher than 30 to 50 per cent of the osmotic values. It is usually less than 25 per cent. The organic acids are unimportant but this is not true of the various salts, organic and, to a certain degree, inorganic ones. The electrolyte content of crop plants may be increased by potassium fertilizers. In any case, by such application an increase of the osmotic values is produced without a parallel change in water conditions (47).

Even more chlorides accumulate in the cell sap of halophytes. A comparison of the osmotic values of halophytes at a time when the salt content of the soil is increasing shows a corresponding increase in the osmotic values of the cell sap. In this case the increase is almost entirely caused by the increase in the proportion of chlorides alone. In one such case in the marsh regions of the east coast of the United States, an increase in the osmotic values of *Salicornia europaea* from 31.9 to 43.4 atm. was found by Steiner (48). The partial pressure of the chlorides rose from 27.8 to 38.3 atm., while that of the other osmotically active substances remained practically unchanged. The same applies to *Juncus gerardi*. While its total osmotic value rose from 22.0 to 33.3 atm., that of the chlorides changed from 10.5 to 20.2 atm., but that of the other substances altered inconsiderably only, namely from 11.5 to 13.1 atm.

It is known that through an increase in the maximal swelling capacity only, and without altering the hydrature, the action of electrolytes is entirely different from that which is induced by proper decrease of hydrature. As electrolytes influence hydration according to their position in the lyophilic series the effects depend upon the nature of the electrolyte. Therefore, it is important whether halophytes store chlorides in their cell sap or sulphates, as the latter do not increase but decrease hydration. The problems of halophytes for that reason are entirely different from those of xerophytes. With the latter, the problem is water economy and the effects of hydrature; with halophytes it is salt economy and the specific effects of electrolytes accumulated in the cell sap.

With halophytes, therefore, a certain increase in osmotic value in more salty habitats does not, as might be expected according to the established decrease in hydrature, retard the development but rather promotes it (49). True halophytes need a certain amount of salt for their development. Planted in normal soil, they seize upon traces of chlorides which are present and accumulate them in their cell sap. The sugar beet which is derived from a halophilic species is a typical example. Even on the best agricultural soils it stores in its leaves a relatively large quantity of chlorides.

Formerly it was assumed that salty soils, through the osmotic effects of

the salts, are physiologically dry for plants. But this osmotic effect is balanced by the intake of the salts. The concentration of salts in the soil solution being not too high, over-compensation appears to take place, i.e., the concentration of salts in the cell sap may be somewhat higher than in the soil. Only when the concentration of salts in the soil solution is extremely high is compensation not effected. Then a reduction of the water intake and, therefore of the growth of the plant, takes place.

It appears that an increase in the succulence of the organs of halophytes is produced by the salts stored in the cell sap, succulence being a characteristic of all chloride-storing halophytes. This assumption is supported by the existence of a group of halophytes which are known to accumulate numerous sulphates in their cell sap in addition to chlorides. It is characteristic of these halophytes that they are seldom, or almost never, succulent. On the salty soils of the Namib Desert of Southwest Africa succulent and less succulent halophytes were observed growing together. Analysis of succulent halophytes gave a ratio of chlorides to sulphates in their cell sap of about 300:1; that of less-succulent halophytes, possessing succulent leaves but nonsucculent stems gave ratios of 30:1 to 11:1 only. In some nonsucculent halophytes which, in part, were able to excrete salt through the leaves, the sulphate proportion may even surpass that of the chlorides. As sulphate ions, in contrast to chloride ions, exert a strongly dehydrating action on proteins, the lack of any considerable amount of succulence may be attributed to the specific effect of the sulphates.

It appears, therefore, that succulent chloride-halophytes must be distinguished from nonsucculent sulphate-halophytes. To this explanation van Eijk (50) made the objection that no increase in succulence could be observed when *Salicornia* was grown in a solution rich in nitrates, although, according to the lyophilic series nitrate ions are even more hydrating than chloride ions. Here van Eijk overlooks the fact that it is not the concentration outside the plant but that within it, namely, in the cell sap, which is decisive for the morphological effect. Van Eijk did not examine the nitrate ion concentration of the cell sap. It is very improbable that nitrates accumulate the way chlorides do for nitrates are food materials and, in the metabolism of a plant, are consumed in the synthesis of proteins; therefore, they cannot directly affect the hydration of the protoplast.

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WATER RELATIONS OF PLANT CELLS AND TISSUES[1]

By Paul J. Kramer

Department of Botany, Duke University, Durham, N. C.

This review deals principally with papers which have been published during the 5 years since the review by Kramer & Currier (69) appeared, but a few older papers have been cited to provide background material. No attempt was made to cite all papers published during this period, but an effort has been made to show the fields in which research has been active. Readers are referred to the reviews by Bogen in recent volumes of the *Fortschritte der Botanik* for additional literature and another viewpoint concerning cell water relations.

In the review by Kramer and Currier considerable space was devoted to a discussion of the possiblity that the classical concepts of osmotic phenomena are inadequate to explain fully cell water relations, and that some active or nonosmotic uptake of water may occur. The occurrence of nonosmotic water uptake remains one of the most interesting problems in the field of water relations of cells and tissues, but although a number of interesting and sometimes conflicting papers have appeared, it is still difficult to reach definite conclusions concerning its importance.

A few years ago Steinbach (111) wrote that studies on permeability were being replaced by studies on accumulation and studies which stressed the dynamic characteristics of plant membranes. This probably is generally true, but several interesting papers on factors affecting the premeability of plant cell membranes have appeared in recent years. Even the old problem of terminology, which some of us hoped was satisfactorily settled by the diffusion pressure deficit concept of Meyer (85), has been revived by several workers who prefer some other term. As Walter (121) wrote, "Fast jeder Autor benutzt ein andere Bezeichnungsweise und mitunter widerspricht er sich selbst." While this reviewer does not recommend slavish adherence to custom he doubts if much has been contributed by recent attempts to revise our terminology.

Terminology and Theory

Broyer has continued his mathematical treatment of osmotic phenomena in a series of three papers. The first paper (25) gives a mathematical analysis of water and solute movement in two-phase systems. In the second paper (26), he discusses the various terminologies which have been proposed and points out some of their limitations. He prefers to use "specific (volumed) free energy" rather than diffusion pressure deficit, and expresses the forces

[1] The survey of the literature pertaining to this review was concluded in October, 1954.

responsible for water movement as follows: $NIF = \Sigma IF - \Sigma EF$, or net influx specific free energy = sum of all energy intensities causing influx—sum of all energy intensities causing efflux. Broyer concluded that concentration and pressure are the important factors in water movement, temperature and surface forces being relatively unimportant and the role of metabolic and electrical forces debatable. He also concluded that a component of a solution always tends to move in the direction in which its specific free energy decreases, both where only differences in concentration are involved and also where pressure, temperature, electrical, or metabolic forces are involved. The third paper (27) is a theoretical one, dealing with methods of computing the coefficient of enlargement of osmotic systems such as cells.

Considerable discussion resulted from a paper by Burström (29) in which he used the term "turgor pressure" in the same sense that the term "diffusion pressure deficit" is used to describe the force causing the entrance of water into cells. It is confusing and undesirable to use a familiar term in a new manner and, as Broyer (24) stated, unnecessary. Burstrom further stated that cell enlargement could occur only if turgor pressure (as he defined it) exceeded wall pressure. This led to a series of papers by Algeus (2), Haines (55, 55a, 55b), Hygen & Kjennerud (65), Spanner (109a), Thoday (114, 115), Weatherley (121a), and others dealing with the relationship between wall pressure and turgor pressure (in the usual sense) in enlargement of cells. These papers are more confusing than enlightening to the casual reader, but consideration of the problem with which they deal suggests that reconsideration of the role of turgor pressure in cell enlargement is necessary.

Some confusion has developed because of the general assumption that turgor pressure causes cell enlargement. If this were true then cell enlargement could occur only when turgor pressure exceeds the resistance of the wall to extension, but turgor pressure is not the cause of cell enlargement. Enlargement of mature cells occurs because water diffuses into them with a force sufficient to stretch the walls. The energy causing cell enlargement is that which causes water to diffuse into the cell; that is, the difference between the diffusion pressure of the water in the cell and the water in the environment, expressed as the diffusion pressure deficit of the cell. Turgor pressure is merely the pressure developed in the protoplast as a result of the increase in volume caused by the entrance of water, and wall pressure is developed because of the resistance of the wall to expansion. Both wall pressure and turgor pressure are produced by the increase in volume of water in the cell and are always equal to each other whether the cell is increasing or decreasing in size or is in a state of equilibrium.

In brief, cell enlargement in mature tissue is caused by the inward diffusion of water along a diffusion pressure gradient, as has been pointed out by Hygen & Kjennerud (65), Haines (55a), and others. Burström (29) also intended to show this, but obscured his point by using turgor pressure in a new meaning. Enlargement of growing cells is more complex because it is

intimately related to metabolic activities resulting in the deposition of new cell wall material and enlargement of the wall (113a).

Levitt (74) objected to Meyer's terminology because it uses three symbols to represent a single quantity (*DPD*) where only one should be used to conform with the usage of chemists and physicists. He would use osmotic pressure as a generic term, applying it to all pressures resulting from osmosis, such as turgor pressure, wall pressure, and potential pressures of solutions. Levitt used the term $D_c - D_{H_2O}$, instead of *DPD*, for the diffusion pressure difference between the cell and pure water because it more clearly indicates what is being compared. In this statement D_c is the diffusion pressure of water in the cell and D_{H_2O} is the diffusion pressure of pure water. In a later paper Levitt (75) proposed that we speak of the "osmotic equivalent" of a cell, which he defined as the osmotic potential of a solution in equilibrium with the cell, or the equivalent of *DPD* in the Meyer terminology. Water moves from cells with low osmotic equivalents to cells with high osmotic equivalents and the osmotic equivalent of a cell can be expressed as follows: $E_c = O_c - P_c$, where E_c is the osmotic equivalent, O_c the osmotic potential, and P_c the wall pressure. Walter (121) also objected to the use of two or three letters to represent a physical quantity and apparently prefers to use *S*, the abbreviation for Saugkraft.

Although there are some disadvantages in the diffusion pressure terminology it seems doubtful if any of the alternatives proposed thus far offer sufficient advantages to justify changing to them. The specific free energy concept of Broyer is difficult for students to understand who have not had considerable training in mathematics and physics, and the terminology of Levitt probably is no easier to understand than that of Meyer. Perhaps these efforts to develop a new terminology will eventually result in something simpler and more logical than what we now have, but this has not yet been achieved.

Water Balance Within Cells

Several papers have appeared which give additional information concerning the distribution of water within the cell. Frey-Wyssling's recent book on the structure of protoplasm (46) emphasizes the importance of physical factors in the formation of vacuoles and distribution of water among the various parts of the cell. He regards protoplasm as consisting of three principal phases, water, proteins, and lipids, in rather unstable equilibrium with each other. According to this view, which, incidentally, has been criticized by Lepeschkin (72), vacuoles may be formed by separation of the water phase as in coacervates, and some substances accumulate in vacuoles because their concentration exceeds that which can exist in the cytoplasm. Although physical factors undoubtedly play a dominant part in the distribution of water among the various structures of a cell, metabolic factors are also important, either directly or indirectly.

It is generally agreed that nuclei and plastids are surrounded by differ-

entially permeable membranes. For example, Abele (1) recently reported that plasmolytic studies showed that the nuclei of certain plant cells are surrounded by elastic, differentially permeable membranes which are impermeable to sucrose. It has been suggested recently that mitochondria are also surrounded by definite membranes which are differentially permeable (44, 89, 108). Thus, even the smaller structures within cells appear to behave as osmotic units.

The various structures within a cell obviously tend to approach equilibrium with respect to the free energy or diffusion pressure deficit of the water in them. This balance seems to be rather unstable and easily disturbed. If the concentration of solute is changed by translocation, use in respiration, conversion of sugar to starch or vice versa, or of nitrate to protein, changes in diffusion pressure also occur, leading to redistribution of water within and among cells. Lundegårdh (82) has discussed the effects of such changes in wheat roots. Vacuoles seem particularly sensitive and sometimes lose water to the surrounding cytoplasm and contract following immersion of the cells in dilute solutions of neutral red and other substances. Sometimes they appear to solidify and break up (71). Bogen (13) recently reported that vacuolar contraction and cap plasmolysis seem to be related. It is not yet understood why the cytoplasm suddenly is able to take up water from the vacuole. Kenda & Weber (68) reported instances of rapid vacuolar contraction following addition of acid, which they attributed to the presence of pectic substances in the cells suddenly undergoing changes in hydration.

For many years there has been discussion concerning the distribution of water among the various parts of a cell. Mason & Phillis (83) estimated that only 30 per cent of the total water in cotton leaves occurred in the vacuoles, but the method used was questionable. Scarth & Levitt (103) estimated that the vacuoles occupy 42 to 58 per cent of the volume of cortical parenchyma cells in apple twigs, and Crafts, Currier & Stocking (37) stated that the vacuole often occupies 70 to 90 per cent of the total cell volume in storage parenchyma. Butler (32) recently estimated that in young wheat roots 70 to 75 per cent of the water by weight occurs in the vacuoles, 5 per cent in the cytoplasm, and 20 to 25 per cent in the walls. The water in the walls forms a continuous system throughout the root, that in the cytoplasm probably is continuous also, but the water in the vacuoles is, of course, discontinuous. It is assumed that the water in the cytoplasm is continuous because of the presence of plasmodesmata which are believed to extend through the walls of most or all cells, connecting adjacent protoplasts by strands of cytoplasm. These interconnected cells are sometimes termed a symplast or symplasm and Arisz (3, 4) thinks that movement of solutes from cell to cell occurs through the symplast rather than by free diffusion through the walls. Presumably a limited amount of water might move from cell to cell along the same path. Lambertz (71a) recently reported that plasmodesmata even occur

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in the outer walls of epidermal cells. Evidently their role in physiological processes deserves more attention than it has previously received.

Changes in Water Content

The total water content appears to be lower in meristematic tissue than in cells which have enlarged. Kramer & Wiebe (70) found the water content of the apical 2 mm. of barley roots to be less than that of the region 2 to 50 mm. behind the tip. Unpublished data of these authors indicate a similar situation in roots of loblolly pine (*Pinus taeda*). In meristematic tissue where vacuoles are small most of the water probably occurs in the protoplasm, but as the plant tissue matures the vacuoles enlarge more rapidly than the dry matter increases and the proportion of water increases. Wilson, Boggess & Kramer (122) published data on diurnal variation in water content of roots, stems, and leaves of sunflower plants. As might be expected, the greatest fluctuations in water content occurred in the leaves and the smallest in the roots, but it was somewhat surprising to find the water content of the roots to be so much lower than that of the leaves and stems.

Few contributions have been made to our knowledge of changes in water content during growth and maturation. Burns (28) noted a sharp decrease in water content of tobacco at the time of flower bud initiation, confirming earlier observations. More work ought to be done on this interesting phenomenon.

Some new work bearing on the occurrence of autonomic variations in water content or water relations has appeared. Enderle (41) observed a morning maximum and nightly minimum in growth and turgor of carrot tissue in culture which persisted after four months in darkness. Engel and his co-workers (42, 43) and Heimann (59, 60) observed diurnal periodicity in guttation which probably is related to changes in permeability or other protoplasmic properties. According to Heimann (60) if less than six or 8 leaves were left on detopped plants, light had little effect on the periodicity, but if more leaves were left attached the autonomic rhythm was replaced by a photoperiodically controlled rhythm. The reader is referred to the review by Kramer & Currier (69) for further discussion of autonomic rhythms in water content and related phenomena.

Water Content and Physiological Processes

Changes in water content are usually accompanied by changes in properties of the protoplasm and rates of physiological processes. Effects of change in hydration of membranes will be discussed in the section dealing with permeability. Christopherson & Precht (35) reported that heat resistance of yeast cells was increased by soaking them in hypertonic solutions and decreased by soaking in hypotonic solutions. They did not at first believe that increased heat resistance could be explained in terms of change in free water

content, but in a later paper (36) they seem to attribute increased heat resistance to increase in the proportion of water which is bound. They consider the solvent volume to control enzyme-activated processes, and state that although the solvent volume and free water are not identical they are modified by the same factors. Iljin (66) claims that drought resistance is usually correlated with the presence of small cells containing very small vacuoles.

Although rates of physiological processes usually are decreased by decreased water content this is not always true. Eckardt (40) found the rate of photosynthesis of *Centaurea intybacea* was decreased relatively little by an internal water deficit. He suggested that the protoplasm of this species must be unusually well adapted to withstand partial dehydration without interfering with metabolic processes. Ried (94) reported that lichens from dry habitats showed little change in respiration and photosynthesis when dried to only 1 per cent of their normal water content, but lichens growing in moist habitats showed marked reduction in photosynthesis below respiration when dehydrated. Montfort & Hahn (86) and Parker (90) found that as plant tissue was dried the rate of respiration decreased, then temporarily increased before falling to zero. Changes in chemical composition may affect physiological processes. Conversion of starch to sugar might result in increased respiration in spite of reduced water content. According to Roberts (95) the lower leaf of wheat plants contained more nitrogen when grown under dry conditions than when well watered, and the water content was higher in leaves of plants grown under very wet or very dry conditions than in leaves of plants grown with a moderate supply of moisture. Simonis (107) found that although drought conditions reduced leaf area it often caused increased water content of leaves, presumably because water deficit resulted in modification of protoplasmic structure resulting in increased water-holding capacity. Further discussion of the relation between water content and physiological processes can be found in papers by Huber (64), Levitt (76), and Richards & Wadleigh (93).

Permeability of Cell Membranes

According to older views, permeability of cell membranes was explainable in terms of pore size, solubility, and electrical charges. Much discussion has occurred concerning the relative merits of the Overton lipoid or solution theory, the Ruhland sieve or ultrafilter theory, and the Collander theory which is a compromise between the two. Bogen (11) and Ruhland & Heilmann (102) recently published evidence to show that by taking into account differences in intermolecular forces, the sieve or ultrafilter theory can be made to explain differences in permeability to a wide range of materials including lipid-soluble substances. The reader is referred to Davson & Danielli (38), Frey-Wyssling (46), and Virgin (120) for more detailed discussions of the structure of cell membranes and theories of permeability. In recent years more complex theories have begun to appear which link permeability more closely

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to metabolic activity than to the physical characteristics of cell membranes. For example, Rosenberg & Wilbrandt (98) relate permeability to enzyme activity and the synthesis of carrier molecules, while Goldacre (47) suggests that transport of water and solutes across membranes depends on changes in adsorptive capacity of protein molecules caused by their folding and unfolding.

Permeability to water as well as to solutes is materially affected by changes in metabolic activity as well as by various chemical and physical factors in the environment. This is to be expected because permeability is controlled by the cytoplasm which consists of a complex framework of hydrated protein molecules and even more complex surface layers. The organized structure of this complex system is maintained by the expenditure of energy released by respiration, and is therefore affected directly or indirectly by factors which affect metabolic processes, electrical charge, or hydration.

There has been a renewal of interest in the roles of the plasmalemma and tonoplast in the movement of water and solutes across the layer of cytoplasm lining the walls of plant cells. It is often supposed that most of the resistance to water movement is located in the surfaces rather than in the body of the cytoplasm, but some investigators think that it is a property of the entire layer of cytoplasm rather than of the plasmalemma and tonoplast. Höfler (61) pointed out that permeability to water is affected by the thickness of the layer of cytoplasm, and removal of everything outside of the tonoplast increases the rate of water movement into and out of the vacuole, but destruction of the plasmalemma does not produce any sudden increase in permeability. Seeman (104, 105) found permeability to water affected by salts, pH, and temperature in a manner which indicated that the entire mass of cytoplasm is involved and not merely its surface. The situation seems somewhat different with respect to solutes. Hope & Robertson (63) and Butler (33) doubt that the plasmalemma has any special impermeability to solutes, and they seem to regard the tonoplast as the principal differentially permeable membrane with respect to ions. On the other hand, there is some evidence that the differentially permeable layer, with respect to sugar, may be the plasmalemma (23, 112). Evidently more research is needed before it will be possible to evaluate properly the role of the various cytoplasmic membranes in cells.

The permeability of plant cells varies with their age and their location. Rosene (99), and Rosene & Walthall (100) observed decrease in permeability of root hairs to water as they grew older, and differences in permeability occurring in a single hair, the tip usually being more permeable than the base. Fischer (45) reported that the permeability as indicated by velocity of plasmolysis increased with age in the epidermal cells of nine species which he studied and Url (117) found the epidermal cells much more permeable than the cells lying beneath them in several species of plants. Pirson & Seidel (91) reported that the cells of the lower part of the zone of elongation

of *Lemna* roots show lower permeability than those in other parts of the root. They also found that deficiency of calcium or potassium affected the location of the zone of lowest permeability. According to Brouwer (21, 22), permeability to water is greater near the tip than in the basal region of roots of *Vicia faba* when transpiration is low, but when it is high the permeability of the basal region increases.

It is generally assumed that dehydration of plant tissue is accompanied by decreased permeability to water, but Brouwer found that when the roots of *V. faba* are subjected to a diffusion pressure deficit of a few atmospheres the permeability of the older part to water and solutes was greatly increased. He suggested that the decrease in turgidity of the cells results in increase in pore size in the cytoplasmic membranes and consequently to increase in permeability. Myers (87) claimed that the permeability of plasmolyzed red beet cells was much greater than that of unplasmolyzed tissue. He attributed this to the fact that those parts of the plasmalemma which are pressed against the micelles of the cell wall in turgid cells are blocked and unavailable as paths for water entry, but when plasmolysis occurs this blocking effect is eliminated. It would seem that this effect would be more than offset by the decrease in pore size accompanying shrinkage and dehydration of the cytoplasmic membranes. Myers' observations seem to contradict those of de Haan (50) and Aykin (5), and particularly those of Levitt, Scarth & Gibbs (79) who found that the permeability of protoplasts of onion parenchyma removed from their walls was the same as that of protoplasts left in their walls. Furthermore, permeability of the cells increased during deplasmolysis. Evidently more research will be required to explain these contradictory results. The reviewer would expect plasmolyzed cells to show decreased permeability because of decrease in size of pores caused by shrinkage during loss of turgor, and because of dehydration. Levitt, Scarth & Gibbs (79) attribute most of the increase in permeability during deplasmolysis to the mechanical effects of stretching the membranes as the protoplasts expand. Possibly, when the protoplasts are inclosed in rigid walls their permeability may decrease when in the fully turgid condition, as claimed by Brouwer (21).

Some interesting studies of the effects of various solutes on cell permeability have appeared recently. According to Guttenberg & Beythien (48), low concentrations of indoleacetic acid increase permeability while higher concentrations, which inhibit growth, also decrease permeability. They found that other substances such as low concentrations of ethylene and phenylacetic acid also increase permeability while growth inhibitors such as coumarin decrease permeability. Meinl & Guttenberg (84) concluded that indoleacetic acid affects the protoplasm rather than acting directly on the extensibility of the cell wall, and Guttenberg & Meinl (49) found the effect of indoleacetic acid on plant growth largely independent of pH. Brauner & Hasman (20) published further work indicating that auxin increases the

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permeability of cell membranes, but also emphasized its effects on cell wall plasticity and extensibility through increase in metabolic activity. Pohl (91a) reported that the effect of auxin on water permeability of oat coleoptiles decreases from tip to base of the coleoptile, but there is no definite gradient with respect to the effect of auxin on the suction force or *DPD* of the tissue. Hasman (58) found that 6-amino-undecane greatly decreased water uptake by potato tissue while di-*n*-amylacetic acid increased water uptake. He suggested that the former might inhibit certain stages in repiration and the latter might activate auxin. Iodoacetate also inhibited water uptake.

It is generally assumed that calcium decreases and sodium and potassium increase permeability and this was confirmed by Guttenberg & Beythien (48). Höfler (62) also verified earlier reports that calcium is essential for repair of the plasmalemma of some species after injury by plasmolysis. Seeman (106) made an extensive study of the effects of various salts and nonelectrolytes on permeability to water, mostly on cells of *Salvinia* leaves. He found that, in general, salts tended to increase permeability, although time and concentration were important factors in determining their effects, an early increase in permeability being followed by a later decrease in many instances. Nonelectrolytes such as sucrose and urea always decreased water permeability. He attributed the effects of electrolytes to alteration of the electrical charge on the cell membranes while sucrose dehydrates it, and urea possibly enters into some combination with the membrane which decreases its permeability. Loeven (80) observed a seasonal variation in the effect of salts on permeability of onion epidermic cells, the effect decreasing from autumn to winter and increasing from winter to spring. He suggested that this variation might explain the contradictory results obtained by different investigators.

Any substance or treatment which affects respiration might be expected to affect permeability because, as mentioned previously, the structure of cytoplasm and cytoplasmic membranes is maintained by the expenditure of metabolic energy. Thus, Brauner & Hasman (20) thought that auxin affected water permeability by affecting respiration and it seems possible that respiration inhibitors may also affect permeability and rate of water uptake in an indirect manner. This will be discussed in more detail later.

Changes in pH of the medium affect water permeability of both protoplasmic and nonprotoplasmic membranes. Seeman (104) found that water permeability decreased at pH values above and below pH 7 in several algae and other aquatic plants. Drawert (39) found the greatest permeability of onion epidermis at pH 6.5 to 7.0. He warned that the osmotic effects of buffer mixtures must be taken into consideration to obtain reliable measurements of pH effects by plasmolytic methods and suggested that some differences attributed to pH were really caused by differences in osmotic pressure of the buffer solutions used. Guttenberg & Meinl (49) found the maxi-

mum permeability of *Rhoeo* epidermis at pH 5.4 to 5.8. Härtel (56, 57) reported that permeability of suberized tissues of twigs and cutinized leaf surfaces as measured by rate of transpiration is affected by salts and pH.

Seeman (105) studied the effects of temperature on water permeability of *Salvinia*, *Maianthemum*, and *Convallaria* over various ranges from 17° to 42°C. He found the temperature coefficient to be rather low, varying from 1.3 to 1.6. Little difference was found between the values obtained with electrolytes and nonelectrolytes as plasmolytic agents. The effect of the higher temperatures was reversible, permeability returning to its original value when the cells were cooled down from 42°C., the highest temperature tested. Guttenberg & Meinl (49) reported a marked decrease in permeability of *Rhoeo* cells as the temperature was decreased below 25°C. Some indirect evidence has appeared which indicates that rapid cooling decreases permeability more than slow cooling. Barney (6), and Böhning & Lusanandana (17) observed that rapid cooling of root systems of several species of plants caused more severe wilting than slow cooling. Perhaps rapid cooling produces a large increase in viscosity and corresponding decrease in permeability, but slow cooling gives time for compensatory reactions which lessen the effect on protoplasmic structure.

According to Seeman (105) irradiation with ultraviolet light increases permeability of the cells to water and the effect persists for 20 to 24 hr. after irradiation in those cells which survive. Virgin (118) found that exposure to visible light decreases protoplasmic viscosity with a simultaneous increase in permeability. This was presented as evidence of the dependence of permeability on protoplasmic structure. Later Virgin (119) found that the effects of light on viscosity can be sharply localized in a narrow band of cells in a piece of tissue, the shaded cells remaining unaffected.

Thus far, most attention has been concentrated on the permeability of protoplasmic membranes, but Casari (34) claims that the cell wall shows differential permeability to large and small molecules. He observed that when plasmolyzed plant cells were transferred from a solution of small molecules to one of large molecules of the same osmotic pressure there was an immediate increase in volume of the protoplasts. When the cells were moved from a solution of large molecules to one of small molecules further decrease in volume of the protoplasts occurred. Bennet-Clark & Bexon (9) observed the same phenomenon and attributed it to the effects of the electrolytes used in the solutions of small molecules on electro-osmotic water uptake. Casari found that removal of the cell wall eliminated the effect of change of plasmolyzing solution and attributed it to more rapid diffusion of small than large molecules through cell walls.

Osmotic and Nonosmotic Uptake of Water

During the past 20 years it has been suggested by several writers that, in addition to the usual osmotic water uptake, there may be active or non-

osmotic water uptake. By active or nonosmotic water uptake is meant the absorption or retention of water against a diffusion pressure gradient by the expenditure of energy released in respiration. According to this concept, water is accumulated by cells much as ions are accumulated. The earlier work was reviewed by Kramer & Currier (69), and Bogen (12, 15) has recently reviewed the literature, but some mention of certain earlier papers is necessary to supply background for the present review.

It was found by several investigators that the osmotic pressure of the solution required to prevent water absorption, as determined plasmolytically, was considerably higher than the osmotic pressure of the cell sap as determined cryoscopically. It was found also that auxin increased water uptake while respiration inhibitors decreased it. All of these findings were interpreted as indicating that nonosmotic uptake of water occurs. Levitt (73) energetically attacked this conclusion, claiming that the discrepancies between cryoscopic and plasmolytic values were the results of errors in measurement, and the effects of auxin and respiration inhibitors were indirect rather than direct effects on water uptake. He calculated that the energy required to maintain an active or nonosmotic gradient was so great that it would be impossible to maintain a gradient of more than 1 or 2 atm. Bennet-Clark (8), however, claimed the permeability value used by Levitt was too high and his conclusion concerning the energy requirement was therefore incorrect. A lively discussion resulted and it is difficult to present the views of the investigators in the limited space available.

Myers (87) claimed that the permeability of plasmolyzed beet cells is about 20 times greater than that of unplasmolyzed cells, which is contrary to the experience of most other investigators. Levitt (77) attributes this apparent difference to the time lag required for water movement through the slices of tissue used by Myers, rather than to differences in permeability. Spanner (109) also attacked Levitt's conclusions, claiming that he used too large an area in his calculations, that his permeability factor was too high, and that the method of calculating the energy consumption was unsound. Levitt (77) seems to have answered these objections satisfactorily, although the reviewer can scarcely agree with his conclusion that "there is not a shred of evidence to indicate the occurrence of active or nonosmotic water uptake." Spanner (110) then attempted to show by use of thermodynamic principles that a large gradient of nonosmotic water uptake is possible and he claimed that permeability can vary without change in structure of the membrane. This conclusion seems contrary to the usual concept of permeability as a property of the membrane and raises questions concerning the applicability of the equation used by him. Levitt (78) claimed that an error occurred because Spanner used an equation for irreversible processes which would permit calculation of the energy changes when a gradient disappears, as by diffusion, whereas he should have used an equation applicable to maintenance of the steady-state in open systems. If water enters only by diffusion an equilibrium

will finally be attained, but if active absorption occurs a steady-state will exist between diffusion of water out of the cell and nonosmotic or active water intake. Perhaps this illustrates the difficulty of attempting to apply the principles of thermodynamics to plant water relations before we know enough about the nature of the plant processes to use them effectively.

Prell (92) found evidence of nonosmotic water uptake in his study of absorption by diploid and polyploid plants of several species. Bogen & Prell (16) found that the amount of plasmolysis of epidermal cells of *Oenothera franciscana* stems was less than expected from calculations of the change in volume and the osmotic pressure at incipient plasmolysis. They concluded that water uptake by plant cells represents the combined action of three forces: (*a*) osmotic uptake by diffusion, controlled by the concentration of solutes in the vacuole; (*b*) metaosmotic uptake by diffusion, resulting from binding of water by cell colloids; and (*c*) nonosmotic absorption involving transport caused by energy released by respiration. The bound water was regarded as osmotically inactive because it could not act as a solvent. The distinction between osmotic and metaosmotic absorption seems unnecessary because in both instances water moves along gradients of decreasing free energy, and, furthermore, the osmotic and metaosmotic forces must tend to come into equilibrium within each cell. Bogen (14) found that epidermal cells of *Rhoeo* and *Taraxacum* kept plasmolyzed for many hours in sucrose solution showed expansion of their protoplasts, at least above 25°C. This was prevented by sodium azide and dinitrophenol, but was not affected by KCN or indoleacetic acid. Bogen concluded that the increase in volume which he observed in plasmolyzed cells was caused by nonosmotic water uptake different in nature from that previously reported because it was not affected by indoleacetic acid or KCN. He suggested that several types of water uptake occur in addition to the classical osmotic uptake, including electroosmotic uptake, the anion respiration uptake of Lundegardh (82), the indoleacetic acid-sensitive uptake of Hackett & Thimann (54), and the indoleacetic acid-insensitive uptake observed by himself. It is possible that more than one mechanism is involved in water uptake, but it is very difficult to prove their existence.

Respiration and water uptake.—If uptake of water occurs against a diffusion pressure gradient (nonosmotic or active uptake) the energy must be supplied by respiration. Aerobic conditions have been found essential for maximum water uptake in plant tissue by several early investigators and more recently in root hairs by Rosene (100). Respiration inhibitors have also been shown to interfere with water uptake by various kinds of tissue, including potato tuber (53, 54), and epidermal cells of *Rhoeo* and *Taraxacum* (14). Bonner, Bandurski & Millerd (18) proposed that water intake and respiration are linked by a mechanism in which energy is supplied by adenosinetriphosphate and the auxin molecule acts as a carrier.

Although there clearly is some correlation between respiration and water uptake the exact nature of the relationship has not yet been definitely established. There may be direct expenditure of energy to bring about nonosmotic accumulation of water as claimed by Bonner, Bandurski & Millerd (18), or respiration might be related indirectly through effects on permeability of cell membranes, extensibility of cell walls, and cell enlargement. Lundegårdh (82) relates water uptake to respiration through ion uptake, the water either being carried along with the ions or moving along an osmotic gradient produced as a result of salt accumulation. Water uptake has been observed, however, under conditions where uptake of solutes could not be involved (19, 51).

It was mentioned earlier that attempts to calculate the energy required to maintain active water uptake in plant cells resulted in contradictory results. Levitt (73), for example, claimed that it is impossible for a cell to provide enough energy from respiration to maintain continuously a nonosmotic gradient of more than 1 or 2 atm. while Bennet-Clark (8), Spanner (109) and others claim that it is easily possible. Lovtrup & Pigon (81) calculated that the amoeba studied by them carried on active uptake of water equal to 2 to 4 per cent of its volume per hour, and this required only a negligible part of the energy released by respiration. It is likely that if active water uptake does occur it is small in volume and occurs against a small pressure gradient, hence the energy requirement probably would not be prohibitively high. Bogen (15) suggested that if nonosmotic water uptake occurs by means of carrier molecules, relatively little additional energy would be required because the carrier molecules probably are normal products of cell metabolism.

The role of auxin.—It was shown many years ago that low concentrations of auxin increases the uptake of water by plant tissue and additional examples have recently been reported. Hackett (51) found that although water uptake of potato tissue is increased by indoleacetic acid much larger increases are produced by naphthaleneacetic acid. Bonner, Bandurski & Millerd (18) found that indoleacetic acid greatly increased water uptake by slices of artichoke (*Helianthus tuberosus*) tubers. T'so & Steinbauer (116) found that auxin increased water uptake by segments of pea stem, but maleic hydrazide reduced the auxin effect about 50 per cent. Hasman (58) found that di-*n*-amylacetic acid increases aerobic water uptake by potato tissue, perhaps because it activates auxin. It has been found by all investigators that water intake is increased by auxin only under aerobic conditions.

Among the more plausible explanations offered for the increase in water uptake caused by auxin are (*a*) increased uptake of solutes by the cells; (*b*) increased permeability of protoplasmic membranes; (*c*) increased extensibility of cell walls; and (*d*) direct stimulation of active or nonosmotic water uptake. The first explanation was shown to be incorrect several years ago and, recently, Hackett (51) and Brauner & Hasman (19) corroborated earlier

findings that there is no increase in concentration of solutes, but actually a decrease during auxin-induced water uptake. It seems to have been adequately demonstrated that auxin causes an increase in permeability of cell membranes to water, and Meinl & Guttenberg (84) claimed that the increased permeability permits increased entrance of water and increased cell expansion. As mentioned earlier, Bogen (12) pointed out that increase in permeability will only increase the rate of water entrance, but cannot by itself change the amount of water finally absorbed. A change in permeability to water without change in concentration of solutes does not affect the water content at final equilibrium, but only the time required to attain equilibrium.

The effects of auxin on the extensibility of cell walls seem as well established as its effects on protoplasmic permeability, and increased extensibility of cell walls will certainly permit increased water intake by reducing wall and turgor pressure in the expression $DPD = OP - TP$. In an earlier paper Brauner & Hasman (19) concluded that the increase in water uptake caused by auxin during the first few hours resulted from increased permeability, but after 24 to 48 hr. the increased extensibility of the cell walls became the most important factor. In a later paper (20) they concluded that decreased wall pressure, resulting from increased extensibility is the important factor in auxin-induced water uptake and were unable to identify a nonosmotic component. Burström (30) emphasized the importance of auxin in increasing the extensibility of walls of growing cells and Levitt (77) attributed the increased uptake of water in the presence of auxin to the increased extensibility of the cell walls. According to this view, so long as the walls are extending plastically osmotic water uptake continues because the turgor pressure never becomes equal to the osmotic pressure. In a recent paper Frey-Wyssling (46a) stated that turgor pressure does not play an essential role in cell enlargement. If this is true the effect of auxin is exerted principally through increased deposition of cell wall material and increased water intake is the result of cell enlargement rather than the cause.

The evidence for a direct stimulation of "secretion" of water into the vacuoles of cells is even more difficult to evaluate. A recent series of papers by Hackett and Thimann deal with various aspects of this problem. Thimann (113) once suggested that auxin causes "the direct metabolic pumping of water" into the vacuoles of cells, but Hackett & Thimann (54) concluded that water uptake is not directly linked to respiration because the two processes are not affected to the same extent by auxin and respiration inhibitors. Water uptake was inhibited more than respiration by respiration inhibitors and it was increased more than respiration by naphthaleneacetic acid, while dinitrophenol increased respiration and decreased water uptake. Bonner, Bandurski & Millerd (18) found that water uptake by artichoke tissue was increased by indoleacetic acid and occurred from solutions apparently having an osmotic pressure greater than that of the cell sap of

the tissue. This auxin-induced water uptake was stopped by the same concentrations of dinitrophenol which inhibit oxidative phosphorylation. Their data indicated that addition of auxin increased the rate of respiration only if water was absorbed, suggesting the existence of a "water respiration" analogous to the so-called "salt respiration" associated with the uptake of ions.

Burström (31) repeated some of the experiments of Bonner, Bandurski & Millerd and concluded that they did not demonstrate nonosmotic water uptake. Part of the supposed nonosmotic uptake was attributed to unavoidable errors in measuring the osmotic pressure of the artichoke tissue at incipient plasmolysis and there also was absorption of the mannitol used as the plasmolyzing agent. Burström claimed that all of the water absorption which occurred with or without indoleacetic acid can be accounted for by osmosis and nonosmotic water uptake does not necessarily occur in artichoke tissue.

Although these investigations have demonstrated a relationship between auxin, respiration, and water uptake they have not proven conclusively the occurrence of nonosmotic water uptake. Most of the experiments ran for several days and growth or permanent enlargement of the cells probably occurred. This certainly was true of the potato tissue used by Hackett and Thimann and the water uptake which they observed was, as they stated, therefore associated with growth or permanent enlargement of cells. Thimann (113a) has recently concluded that the auxin-induced uptake of water studied by Hackett and Thimann occurs because auxin stimulates cell wall extension and is not an example of nonosmotic water uptake. Burström (30) has commented on the difficulty of identifying a nonosmotic factor in water uptake by growing cells because the wall pressure is continually changing. He found no evidence of the existence of a nonosmotic component in water uptake by growing wheat roots. There is not yet sufficient information available to explain fully the relationship of auxin to water uptake, but it probably affects it indirectly, perhaps by affecting metabolic processes which in turn affect the extensibility of cell walls. The effects on permeability probably are of secondary importance and the effects on direct secretion of water into the vacuole cannot yet be evaluated.

Mechanisms for active uptake of water.—Thus far, no satisfactory mechanism for active or nonosmotic uptake of water has been proposed. Interest in electro-osmotic mechanisms seems to have decreased, the only recent paper proposing one being that of Bauer (7), who found that the osmotic pressure of a calcium chloride solution required to produce incipient plasmolysis was slightly lower than that of the sucrose solution required to produce the same effect. Bauer explained this as resulting from inhibition of the electro-osmotic factor in water uptake by $CaCl_2$, but it seems just as probable that the decrease occurred because $CaCl_2$ produced changes in water binding capacity and hydration of the cells. Blinks & Airth (10) found that applied

electrical potentials up to 1500 mv. produced no appreciable movement of water into *Nitella* cells. It is probable that electro-osmosis plays no important part in the absorption of water by plant cells, although the possibility of limited movement by this means can scarcely be excluded.

Most theories of secretion are extremely hazy with respect to the details of their operation, although at the present time, the idea of "carrier" molecules is popular. Goldacre (47), for example, proposed that protein molecules unfold at one surface of a membrane, presenting a large surface on which ions and water molecules are adsorbed. The molecules are carried across the membrane, perhaps by protoplasmic streaming, and fold on the other surface with consequent desorption of the material they are carrying. Rosenberg & Wilbrandt (98) propose a more complex theory of transport which postulates enzymatic formation of carrier molecules on one surface of the membranes and their destruction on the other surface, where the material they are carrying is released. Bogen (14) has recently reviewed the literature on absorption of materials by cells.

Workers in animal physiology would be expected to contribute to our understanding of secretory processes. Although they have often assumed that water movement in animals could be explained by osmosis, according to recent reviews (67, 96, 97) they are beginning to doubt this. Unfortunately, they do not seem to have any more satisfactory theories to explain nonosmotic movement than the plant physiologists. Robinson (96) thought that kidney cells might secrete water on the basal margin because of higher metabolic activity in that region. This recalls the suggestion of Osterhout (88) that if a plant cell maintains a higher concentration of solutes at one end than at the other by expenditure of metabolic energy it might absorb water at that end and expel it at the opposite end.

Conclusions

Although worthwhile additions have been made to our knowledge of the water relations of plant cells and tissues, disturbing gaps still exist. No satisfactory mechanism for active or nonosmotic water uptake has been described and, in the opinion of many physiologists, the occurrence of nonosmotic water uptake has not even been demonstrated conclusively.

There seems to be little doubt that osmotic movement of water along diffusion pressure gradients accounts for most of the changes in water content of plant tissue. In mature tissue these changes often are large, amounting to 10 per cent or more of the fresh weight, they often occur very rapidly, and they are reversible. In growing tissue the changes in volume of water usually are much slower and largely irreversible, but even water uptake by growing cells is largely or entirely explainable by osmosis. Many of the supposed examples of nonosmotic water uptake probably can be attributed to difficulties in measuring the osmotic quantities of plant cells, but there are

a few instances of apparent nonosmotic uptake of water which are not easily explained in this way. It seems possible that nonosmotic uptake of water occurs, but if so it is small in quantity and probably of little importance in plant water relations. It certainly is very difficult to demonstrate conclusively.

Perhaps further research will necessitate modification of the present view. The problem of active or nonosmotic water movement certainly deserves further attention, particularly with respect to development of a theory which would adequately explain nonosmotic movement of water across membranes.

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COLOUR DEVELOPMENT IN FLOWERS[1]

By Karl Paech[2]

Department of Botany, University, Tübingen, Germany

Introduction

Flowers are a rather late acquisition in the process of plant evolution. Although doubts may be justified, and arguments may go on, flowers are considered to be homologous to, and derived from, vegetative shoots; they are not considered to be organs *sui generis*. The question, therefore, arises whether the metabolic activities of flower parts are specialised functions of normal leaves or whether the flower colours result from singular abilities of the calyx, petals, etc.

The production and the accumulation of pigments other than chlorophyll are not essential functions of flowers, as such. The primitive types of flowers produced early in the course of evolution had not yet developed relationships with insects or other animals with light-sensitive organs. It was only a further step in evolution, a more modern "invention," which resulted in flowers attracting animals for pollination. It is noteworthy, too, that those flower parts which develop colours other than green ("Schauapparat"), are not confined to the petals, calyx, and other organs of the flowers themselves, but are frequently joined by bracts which are closely related to green leaves. On the other hand, colours are not exclusively developed where they serve the purpose of attracting animals. The bright yellow anthers of some conifers and Gramineae and the red stigma of *Corylus avelana* are conspicuous examples of purposeless colours. Colour development is not necessarily connected with flower formation and flowers exhibit colours other than green even when there is no detectable use of them.

The colours of organisms can be produced in two quite different ways: either by the refraction of light on physical structures and surfaces, or by the absorption of definite wave lengths by pigments. In the plant kingdom the latter type predominates. White colour, however, normally does not result from a pigment, as, e.g., in the white birch periderm, but originates from total light reflection on surfaces and interfaces. This mechanism is at the disposal of all plant organs which contain intercellular spaces or air-filled dead cells. In tissues free of pigments the unchanged light is reflected by the surface between cells and intercellular spaces so as to confer a white colour on the tissues concerned. Other minor phenomena which contribute to the actual shades of flower colours will not be discussed in this review.

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Which are the colours, apart from green, that are known to occur in foliage leaves? The yellow coloured leaves and leaf portions of aurea or panachured varieties, e.g., of *Sambucus nigra* and *Acer negundo*, exhibit the carotenoid colour normally masked by chlorophyll. In these cases the reduction or absence of chlorophyll is due to a genetic block or loss. If the production of carotenoids, too, is inhibited, the conspicuous albino leaf effect is obtained. So far, colour changes in green leaves are essentially connected to plastid pigments. In the cell sap of leaves flavonoid compounds are common constituents, though not all are coloured under the conditions prevailing in living cells. A great number of flavones and flavonols, leucoanthocyanins, and catechins are colourless, but chalkones, certain flavones and, of course, anthocyanins exhibit a wide range of colours. Emphasis should be given here to the frequent occurrence in young leaves of anthocyanin which is produced before chlorophyll has been developed in the leaves. The phenomenon of deep red young leaves which turn green during leaf growth is widespread in many families and is particularly conspicuous in the tropics. This applies not only to trees, shrubs and other flowering plants, but also to the red young fronds of many ferns. In the course of plant evolution, the ability of young leaves to produce anthocyanin has been a general feature of leaf metabolism before flowers had appeared. In certain plant species, deep colours other than green may remain even in mature leaves, e.g., in *Begonia* species and *Coleus* varieties. The possibility exists that yellow coloured flavonoids are present in foliage leaves and are masked by chlorophylls and carotenoids but would appear when the production of plastid pigments is inhibited. This problem has not been sufficiently investigated.

Colours other than green in foliage leaves occur at two very different stages of leaf development. First they appear in the very early phase of elongation. Frequently, in this period, appreciable amounts of anthocyanins are produced which fade away as chlorophyll is formed. This is probably because of the activity of an enzyme (62). A highly interesting complementary case with "youth carotenoids" has been reported (72a). Secondly, colour changes occur in senescent leaves when the chlorophyll is destroyed; then other pigments may be produced or, if already present, may appear.

The colour types mentioned so far cover almost all the colours exhibited by flowers. It, therefore, seems highly probable that colour development in flowers is but a specialisation of general leaf metabolism. Since, in biology, common experience teaches that nature uses all possibilities in its effort to "fulfil a purpose," a strong presumption exists that both the very early and the very late phase of leaf development may contribute to flower coloration. This problem will be further discussed in connection with the various pigments.

It should be mentioned that neither betulin nor pigments of the berberine type have been reported in flowers. Phlobaphens, the common bark and seed coat pigments, have not been found in flowers, unless the brown and black pigments in the involucrum of certain compositae are found to be of the phlobaphen type.

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There is, however, evidence of the occurrence in vascular plants of melanins, i.e., dark polymeric N-containing derivatives. There are not many published records of the appearance of melanins in still turgid functioning organs; the black pigment in the wing petals of *Vicia faba* is the best known example (91). There is some probability that melanins can be produced in foliage leaves; this would also seem to connect flower colours with the chemical activities of leaves. There are very few flower colours which do not originate from pigments mentioned so far. The brown pigment of certain saprophytic orchids, e.g., of *Neottia nidus avis*, has not been identified, but here again the whole plants, not only the flowers, are tinted with the same pigment.

Although generally, only similar classes of pigments are produced in flowers and in normal leaves, we must be prepared to find a greater variety of individual compounds in flowers than in leaves, just as the morphological variation of petals and other flower parts is much wider than the modifications of green leaves.

The Pigments

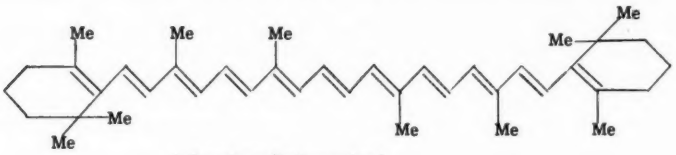
Carotenoids.—Chemically, carotenoids are aliphatic compounds of the isoprenoid type. The other main class of flower pigments, the flavonoids, are aromatics. The pigment character of carotenoids originates in a certain number of double bonds in the molecule (polyenes), the number of which actually determines the tone and deepness of the colour which can vary from pale yellow to deep red. When the hydrogenation of double bonds goes beyond a certain limit, colourless polyenes result which have recently been found in various plant organs including petals and other flower parts. Comparatively large amounts occur in the flowers of *Mimulus longiflorus*. In the petals of *Jacaranda ovalifolia* phytofluene surmounts the β-carotene content [(75), cf. also (99)]. Since the class of natural carotenoids comprises a very large number of individual compounds only some characteristic types can be mentioned here by name and formula.

Valuable lists of the carotenoid pigments found in petals and reproductive organs have been included in Karrer & Jucker's (39) and in Goodwin's (26) books. Since then more compounds have been discovered [cf. (27)]. Seybold's lists (81) give information concerning the presence of carotenoid or flavonoid pigments in yellow flowers.

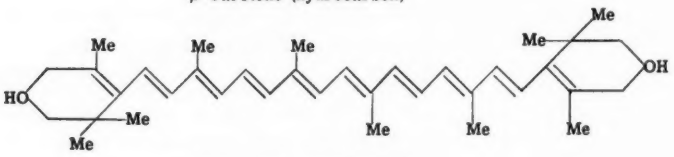
As a consequence of their fat solubility, carotenoids are cytologically, with very few exceptions, plastid pigments. In certain higher and lower plants they occur in fatty oil droplets [lipochromes, cf. (15)]. The location of these pigments in the cell will not be considered here in detail. A comprehensive survey, concluding the great period of anatomical investigations into plant cells, has been given by Möbius (53).

In the petals of *Verbascum phlomoides* and *V. thapsiforme*, the colouring matter is a water-soluble derivative of a carotenoid, namely an α-crocetin glycoside (73). Crocetin, a dicarboxylic acid of the general formula $C_{20}H_{24}O_4$, is not a carotenoid *sensu strictu* but it may well be derived in the cell from a true carotenoid by oxidation at both ends of the molecule via a furanoid

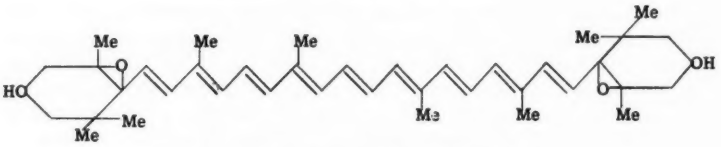
compound (cf. the formulae). When the petals are still in bud they do not contain a water-soluble pigment but "normal" plastid carotenoids which are gone when the petals are fully grown (81). Water-soluble yellow crocetin sugar esters seem to be fairly widespread in the plant kingdom; several closely related crocetin gentiobiose esters occur in the stigma of *Crocus sativus*, the saffron (37). The yellow petals of *C. luteus* contain crocetin and



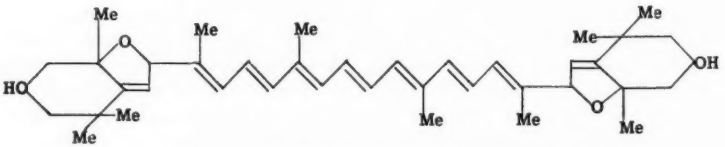
β-Carotene (hydrocarbon)



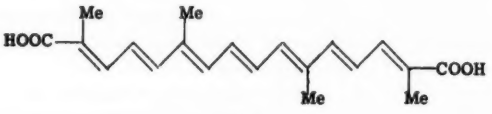
Zeaxanthin (a xanthophyll)



Violaxanthin (= zeaxanthin-di-epoxid)



Auroxanthin (a furanoid derivative of zeaxanthin)



Crocetin

SCHEME 1. Examples of the various types of carotenoid pigments.

it probably is the colouring substance in the flowers of *Cedrela toona*, Meliaceae, and *Nyctanthes arbor tristis*, Oleaceae (40).

Apart from their function of attracting animals in the pollination of flowers, carotenoids in flowers are thought to affect other activities. Some carotenes, e.g., β-carotene, and some xanthophylls strongly inhibit the germination of *Cyclamen* pollen grains, whereas other derivatives, particularly crocetin, exhibit a strong stimulation on pollen germination under experimental conditions (74). Whether this is one of their natural functions remains to be shown.

The carotenoids of mature green leaves consist mainly of several xanthophylls, predominantly lutein, and of β-carotene sometimes accompanied by remarkable amounts of α-carotene. About a dozen individual compounds have been found either in small amounts or in traces only. These carotenoids in green leaves have been termed "primary" ones to denote that they belong to the general category of the chloroplasts (80). During the fading of leaves in the autumn the carotenoids undergo changes which are not yet well known in detail [cf. (26) p. 22, (55)]. These autumn carotenoids, together with the carotenoids produced in flowers and fruits, have been termed "secondary" carotenoids which, however, are not well characterised. Yellow leaves of aurea varieties contain a mixture of carotenoids similar to those of senescent leaves, i.e., the oxidised xanthophylls are in excess [cf. (26), p. 23]. There are some interesting cases of the production of "youth carotenoids." *Adoxa moschatellina* has orange chromoplasts in the young stems and leaves (25). The young proplastids accumulate very little chlorophyll, and mainly carotenoids which disappear during leaf elongation. In two other unrelated species (*Ceratozamia mexicana* and *Haworthia coarctata* var. Krausii) appreciable amounts of an oxidised carotenoid rhodoxanthin occur in the very young leaves which are stained reddish-brown (72a). This "youth carotenoid' disappears in very much the same way that anthocyanins fade during the growth of those leaves which are bright red in the first stages of development (see above). The phenomenon of youth carotenoids seems to be an early achievement in plant evolution since it has been observed in *Selaginella pervilli*, also (54).

The carotenoid pattern in green leaves is rather simple and uniform throughout the plant kingdom. However, when we consider the carotenoid content of petals and other flower parts we no longer encounter any uniformity. A wide variation of individual compounds occurs, some of which are specific to petals, while some are even specific to a plant. Generally, xanthophylls, free or esterified, and epoxides are characteristic of petal carotenoids although no general rule can be given for their frequency or for the mixture they form.

To mention in detail at least one typical example of carotenoid mixture in petals. the analysis of a variety of *Calendula officinalis*, a member of the Compositae which produces dark orange flowers, may be given here (27). The following hydrocarbons have been identified: phytofluene, β-carotene,

γ-carotene, ζ-carotene, lycopene and pigment X, a carotenoid whose exact structure has not yet been established. Apart from these another unidentified hydrocarbon is present, probably prolycopene. Among the xanthophylls the following were found: flavochrome, mutatochrome, aurochrome, flavoxanthin, chrysanthemaxanthin (all furanoid derivatives), lutein, and neoxanthin. Moreover, there are both a rubixanthin-like pigment, and another which has not yet been identified. Quantitatively, carotenes (=hydrocarbons) and xanthophylls (oxidised carotenoids) each represents about one-half of the total pigments present. Lycopene accounts for more than half of the hydrocarbons. This analysis confirms that "5,8-epoxides (=furanoid carotenoids) are widely distributed in petals in relatively large amounts and that lutein, although present, is not, as in green leaves, the major xanthophyll. Furthermore the investigation adds emphasis to the recent observation made on berries (27) that when examined in terms of present knowledge concerning separation and identification of trace carotenoids, the pigment mixture is found to be much more complex than was originally thought. The biological significance of these complex mixtures is at present quite obscure (27). They are, however, matched by similar complex natural mixtures of other types of secondary plant products, e.g., alkaloids.

At the moment there are no known rules concerning the distribution in plant families or genera of carotenoids or flavonoids, the two main classes of flower pigments. In certain families, e.g., Rosaceae, the anthocyanins predominate but even here yellow flowers may contain carotenoids (e.g., *Potentilla* species). Seybold (81) gives many examples to show that in most yellow flowers both carotenoids and flavonoids occur (200 out of 250). Species of the same genus or closely related genera may contain different types of pigments. What was thought to be a rule—that anthocyanin-containing species will be related only to flavonoid yellow flowers—has a very limited validity. Seybold gives about 170 pairs of species, one of which is coloured by carotenoids, whereas the corresponding one is either coloured by flavonoids or has white flowers which generally contain colourless flavonoids.

A rather curious correlation has been observed between the type of flower or fruit pigment and the members of the triterpenoid class of substances occurring in the same plant species. According to the "Zimmermann rule" those species which contain anthocyanins produce triterpenoid acids, e.g., oleanolic acid. Species whose flowers and fruits are tinted by carotenoids preferably produce triterpenoid alcohols, e.g., betulin. In the Compositae family *Arnica, Helianthus, Taraxacum,* and *Tussilago* all have carotenoids as colouring substances in the flowers and all develop triterpenoid diols. Exceptions to the rule are already known: *Calendula officinalis* although containing carotenoid flower pigments, produces oleanolic acid. On the other hand, hips differ from most Rosaceae in containing carotenoids as the main pigment; and actually betulin, a triterpen alcohol, was discovered in them after a search was made when the rule was known (100). At present, there is no known metabolic relationship to explain the observed facts.

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Scarcely anything is known about the biogenesis and metabolism of carotenoids in petals and other flower organs. Since there is no fundamental difference between the carotenoids in flowers and green leaves some observations collected from green or etiolated leaves may be added here. In detached green leaves carotenoids are synthesised in the dark as well as in the light. Their production is greatly increased by the addition of sugars. Although some of the C_3 intermediates of glycolysis are not able to replace the sugar it seems highly probable that carotenoid synthesis draws from the normal carbohydrate breakdown [(1), (26) p. 72]. This picture has been extended by experiments with etiolated leaves which resemble petals as they do not contain chlorophyll. The maximum carotene content occurs at the period of maximum growth rate. Carotenoid production goes on only when an excess of carbohydrates is supplied (and probably broken down). Phosphorylations seem to be essential in the carotenogenesis. The effect of various inhibitors of different steps of carbohydrate breakdown does not suggest that carotenoids are synthesized from glycolytic intermediates or from acetate alone. Members of the Krebs cycle between citric and succinic acid obviously play an important role (10, 11). Interest as to the formation of carotenoids in petals has been mainly focused on the fine structure of special flower carotenoids, e.g., on the epoxides. In the plant cell the oxidation of the parent carotenoids could be affected by a peroxidase system [(26) p. 72, (38)]. *In vitro*, a further conversion of epoxides to furanoid compounds is achieved by acidification to about pH 3. This could well occur in plant cells also, unless the carotenoids are protected against the influence of the sap acids by the plastid membrane and the cytoplasm. However, a change of the cytological conditions during petal growth may be envisaged. Structurally, lutein epoxide could be transformed into flavoxanthin and chrysanthemaxanthin, thus deriving some of the characteristic petal carotenoids from the universal leaf xanthophyll. The epoxides and the furanoid derivatives, though their biological function is not yet elucidated, are thought to be the first steps of carotenoid degradation.

Viola flowers which have developed in spring contain mostly violaxanthin an epoxide, and a little furanoid auroxanthin, whereas flowers which develop in late summer and autumn accumulate increasing amounts of the furanoid compound (38). This is all the more remarkable because the change does not take place in flowers which grow old but in those which are produced on the same mother plant in different stages of development. Since not only the parent plant, but also the season is different, it remains to be shown whether the metabolic change depends on alterations in the plant or on the altered environmental conditions.

It has been reported that the carotenoid content of the anthers and petals of a number of plants increases through the budding period and reaches a maximum at flowering [(26) p. 90]. The petals of *Brugmansia aurea* Saff. (Solanaceae) offer a clear example of the relationship between leaf and petal carotenoids (36). The very young flower tube in the bud is green and contains the typical leaf pigments: chlorophylls together with carotenoids. Dur-

ing elongation the flower colour changes from green to greenish yellow, yellow, and finally orange. At the same time the chlorophylls are degraded and the carotenoid content increases. The mature flower tubes carry large amounts of carotenoid pigments in the epidermal cells, partly distributed as a yellow component in the plasm and partly as needlelike red crystals; the actual colour is a mixture of both pigments.

Emphasis must be laid upon the fact that the development of petal carotenoids in *Brugmansia* is confined to epidermal cells. Seybold (81) describes a similar example, *Chrysanthemum carinatum*, where carotenoids occur only in the epidermis of the petals. Geitler (24) concludes that chromoplasts in yellow and orange petals mostly arise from leucoplasts and only exceptionally from chloroplasts (e.g., in *Ranunculus* and *Tropaeolum*). In the red petals of *Aloe echinata* the epidermis is colourless whereas the parenchyma is filled with small red particles. In *Coelogyne tomentosa* and *Narcissus poeticus* red chromoplasts occur both in the epidermis and in the parenchyma [(53) p. 97]. The question whether the chromoplasts in petals are identical with the chloroplasts in the same young petals cannot yet be unambiguously answered. In some, at least, of the relatively large number of "green flowers" intermediate stages between green leaves and yellow petals may be seen. Approximately 20 per cent of the species of the flora in France and Germany have green flowers [cf. (81) p. 13]. In some carotenoid-coloured flowers appreciable amounts of chlorophyll remain even in the mature stage of the petals, e.g., in *Caltha palustris* and *Forsythia intermedia*. Finally, it may be mentioned that there are petals which contain a lower concentration of carotenoids than the leaves of the same species when compared on a fresh weight basis [(81) p. 20].

In connection with the above mentioned disappearance of red carotenoids from young leaves during elongation, the colour changes of *Kniphofia aloides* flowers should be quantitatively examined (81). Young flowers are coloured red by two pigments which are lycoxanthin and celaxanthin. They are thought to be oxidised to yellow xanthophylls, changing the flower colour to yellow. There is still, however, the possibility that the red pigments disappear without giving rise to new yellow pigments and only unmask the already present yellow carotenoids.

Regarding the behaviour of carotenoids in foliage leaves, the carotenoid-coloured petals could correspond either to aurea leaves, (i.e., in which little or no chlorophyll is developed in the plastids at any time) or to autumn leaves when the chlorophyll is destroyed early in the young green petal plastids. Seybold, who favours the latter view, suggests that the colour changes in petals (and in ripening fruits) have very much in common with the fading foliage leaves in their senescent stages. Unfortunately, the composition of "autumn carotenoids" is still insufficiently known. In tobacco leaves no increase of carotenoids has been found during fading (55). More extended examination of both the cytological and the metabolic side of carotenoid formation in petals is required before an unambiguous decision can be made whether petals actually are equivalent to senescent leaves. If this were so

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there would exist the most interesting, and at the same time puzzling, fact that in the sequence of flower parts senescent petals are located between the relatively juvenile calyx and the almost embryonal carpels since both are capable of further growth after pollination and during postfloration.

Other problems regarding the metabolism of petal carotenoids are obvious. What are the factors causing the oxidation of "primary" carotenoids to crocetin and why does not the oxidation of carotenoids proceed to stages of crocetin or similar water-soluble carotenoid derivatives in other species, too?

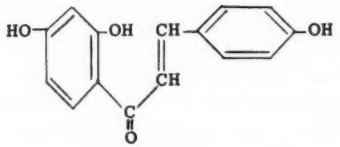
Flavonoids.—The term flavonoids as used in this review includes all compounds possessing a C_6-C_3-C_6 skeleton whether the two aromatic nuclei are connected by an open chain as in the chalkones, or by a heterocycle as in the anthocyanidins, etc. The class comprises manifold different types of which anthocyanins, flavones, flavonols, flavanones, chalkones, isoflavones, and "aurones" (benzylidene coumaranones) should be of prime consideration here because all or, at least, some members of a group are pigments. Other related compounds, e.g., leucoanthocyanins are not pigments themselves but can be transformed into such, probably under cell conditions. They will, therefore, be included as far as metabolic reasons require.

The chemical nature of the chromophoric group in the molecule of the various flavonoid types will not be discussed here. Information can be obtained in the handbooks and textbooks of chemistry. Some modern aspects of the chemical and physical properties of anthocyanidins have been elaborated by Shriner (86). Neither is it proposed to enter into detailed consideration of the numerous flavonoid derivatives and their variations. The matter is dealt with at some length in the textbooks of organic chemistry and plant biochemistry [cf. (13, 49, 61)]. Comprehensive reviews have recently been written by Blank (12), Seshadri (79), Geissman & Hinreiner (23a) [Cf. also (20)]. Brief mention may, however, be made of characteristic members of various types and their main chemical features.

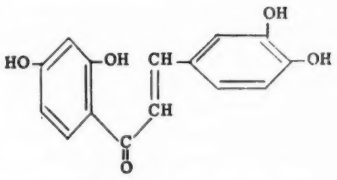
Variation of these types mainly concerns the hydroxylation pattern of the two aromatic rings. Moreover, the hydroxyls may be either partly substituted by —OCH_3 groups or may be glycosidised by various sugar residues of the monose or biose types. In some groups of the flavonoids glycosidation is very rare, while in others it is restricted to certain positions, e.g., with anthocyanidins. As a consequence of glycosidation most of the flavonoids are easily water-soluble. There are, however, glycosides that are only slightly soluble in cold water, e.g., hesperidin.

The colour range covered by flavonoid compounds extends from pale yellow flavones to purple and deep blue anthocyanins. The terms anthochlor and anthoxanthins used to designate water-soluble yellow pigments should be avoided since these substances do not form a class of their own but can be classified in different well-defined groups of chemical compounds: chalkones, flavonols, and carotenoid derivatives as well. As for the colours displayed by the various types of flavonoids the anthocyanins are too well-known to need much mention. An increase in the number of hydroxyl groups causes a

Chalkones

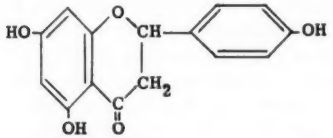


Dahlia chalkone

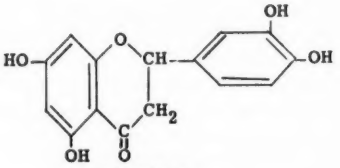


Butein

Flavanones

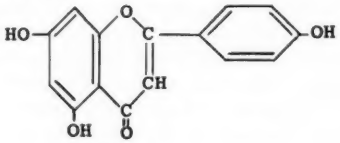


Naringenin

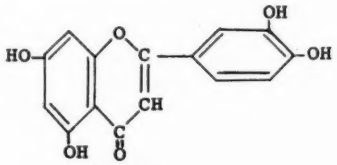


Eriodictyol

Flavones

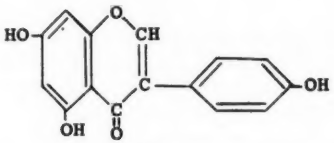


Apigenin



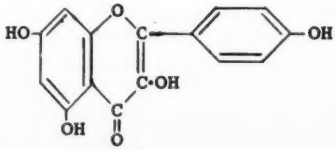
Luteolin

Isoflavones

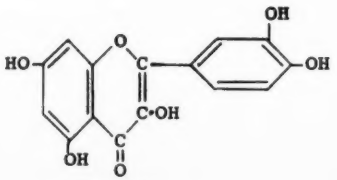


Genistein

Flavonols

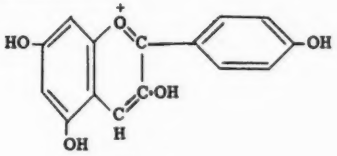


Kaempferol

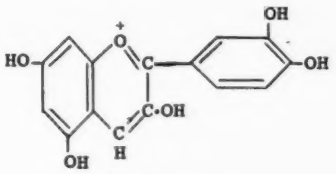


Quercetin

Anthocyanidins

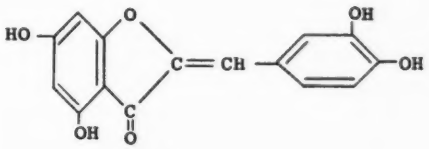


Pelargonidin



Cyanidin

Aurones



Aureusidin

SCHEME 2. Examples of various types of flavonoids.

marked increase in the blueness of tone. The two main classes of glycosides differ in colour, the 3,5-dimonosides being bluer than the corresponding 3-monosides or biosides. Methylated anthocyanins are generally redder than the unmethylated types. Some other factors which modify anthocyanin colours will be dealt with below. The chalkones are yellow to orange in colour depending on the number and the relative position of hydroxyl groups in the molecule. Flavanones and flavanonols are either only faintly yellowish or colourless. At the pH values of cell saps the flavones and flavonols vary in colour from pale to very bright yellow depending upon the degree of hydroxylation, with only the hexahydroxy derivatives and above having any considerable depth of colour. The "aurones" (this convenient term is used for natural benzylidene cumaranones [cf. (5)] are golden-yellow in acid and neutral solutions and deep rose-red in alkaline solutions (3). The first of them, leptosin, was discovered in *Coreopsis grandiflora* flowers (21). Another aurone glycoside, aureusin, is responsible for the colour of yellow varieties of *Antirrhinum majus* which do not contain luteolin as was thought by Wheldale (78). The isoflavones which are of infrequent and irregular occurrence are even more weakly coloured than the flavones.

Flavonoid compounds are common constituents of higher plants and it is indeed difficult to find any flowering plant which does not contain at least one type of the flavonoid class in any of its organs. They are produced and accumulated in plant organs where no advantage or significance of their colours, for the life of the plant, can be imagined, e.g., anthocyanins occur in the root tips of many species. "The presence of anthocyanins may be due to nothing more than the particular metabolic sequence, in other

words, an accident which in some examples may be a lucky one for the plant" (28).

Fundamental differences in the mechanism of anthocyanin production in petals and foliage leaves have been supposed but there is no evidence to prove this assumption. However, as with carotenoids, a greater number of individual members of a certain type is encountered in flowers than is found in leaves, bark, wood, and other parts of the plant. Cyanidin overwhelmingly predominates in foliage leaves whereas in flowers it occurs in less than 50 per cent of the species examined (46). Similar proportions prove true for quercetin among the flavonols.

So far, no evidence has been presented to show whether, in leaves or petals, a connection exists between the metabolically produced carotenoids on the one hand and flavonoids on the other. Seybold (81) suggests that flavonoids are the result of a more advanced cell degradation; the formation of the different pigments being a sequence of metabolic changes which are thought to be characteristic of senescent leaves.

As to the frequency of anthocyanins in relation to climate and geographical distribution, pelargonidin derivatives have been reported to predominate in the flowers of tropical and subtropical origin, whereas delphinidin derivatives were held to be most common in temperate and alpine climates (9a). However, the examination of 165 genera of the flora of Trinidad does not support a high pelargonidin and a low delphinidin frequency in tropical floras (19a). A relation between flower colour and climate may well exist but would seem to be caused mainly by factors other than variation of the anthrocyanidins, e.g., by co-pigmentation, etc., (cf. p. 294). The frequency of methylation of delphinidin is much greater than of cyanidin in the flora of Trinidad; thus, malvidin is a common pigment, peonidin a rare one. The examination of approximately 18,000 species of West European and tropical origin reveals that no remarkable deviation from the average flower colours exists in any area. In almost every area, the white, yellow, and red flowering species number five times as high as the blue species. The alpine flora, with 11 per cent blue flowering species (as compared with 5.5. per cent in other areas), is the only exception (93a). Moving northward in East Asia, the colour of the bracts of certain banana varieties changes from red to deep purple with a concomitant increase in delphinidin and methylated anthocyanidins (87).

Recent contributions to the knowledge of flavonoid flower colours mainly concern (*a*) the discovery of a wider distribution of pigments other than the "classic" ones, and (*b*) the isolation from individual species of series of compounds belonging to different types of flavonoids, which are related in some common features, e.g., in the hydroxyl pattern. These advances have been favoured by the development of chromatographic techniques and absorption spectrophotometry [cf. (1a, 4, 20, 34)]. The distribution and identification of anthocyanin pigments of 74 species ranging from Polypodiaceae to Compositae have been reported by Hayashi & Abe (32). The modern techniques make it possible to make a fair guess on a single chromatogram as to the

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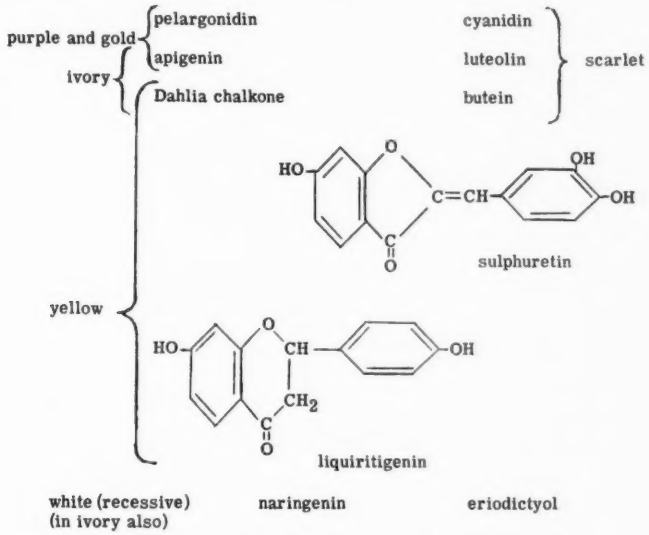
nature and possible structure of unknown pigments in their naturally-occurring mixtures. The introduction of the R_M value in paper chromatography which is log $(1/R_f - 1)$ allows one to deduce the number of hydroxyl groups in unknown aglycones and the number of sugar residues in glycosides related to a known aglycone (4). The application of colour reactions, ultraviolet and infrared spectrophotometry on the spots of a chromatogram in many cases give an absolute characterisation of the structure of the pigment. If a glycoside, the sugars and their position of attachment to aglycones can also be readily ascertained [cf. (56)].

Normally in each species one main flavonoid compound is accompanied by a series of minor flavonoids. There are good reasons for supposing that a close biogenetic relationship may exist between these compounds occurring together. Garden varieties of *Dahlia variabilis* are among the best known examples of this possible co-relationship (43, 45). Recently, Bate-Smith at Cambridge has become interested in the *Dahlia* colours and has advanced the matter to a degree of great clarity. In the different colour varieties of *Dahlia*, several series of compounds are present whose relationships are best demonstrated by the following formulae [cf. (6, 57, 58, 64)]:



SCHEME 3. Distribution of flavonoids in *Dahlia* varieties.
(For missing formulae see Scheme 2).

Some or all of these compounds may be combined with various sugars while some may be acylated to complex anthocyanins. In the dahlias the bulk of flavonoids occurs as glycosides. The multiplicity of derivatives of a few flavonoid compounds in the highly complex genetic pattern can produce

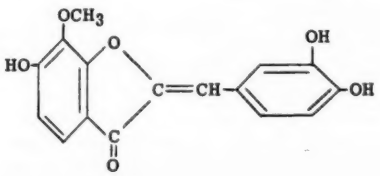
a mixture of chemical individuals, which, on first sight, is confusing, when resolved on the chromatogram. Fortunately the formation of pairs of the compounds is governed by known genetic factors and suitable combinations can be selected to study the pigment complex of a range of simple genetic types (cf. p. 290).

No conclusive evidence as to the possible route of biogenesis of the compounds concerned can be derived from mere comparison of the groups. However, their distribution and co-occurrence suggest that flavanones are intermediates in the flavone synthesis and chalkones in the synthesis of aurones (56). If the genesis of the C_6-C_3-C_6 skeleton implies the condensation of two fragments, one being a polyphenol of the phloroglucinol or similar type and the other a phenylpropane derivative (23a, 61), then each of the elements must be provided in two different types. The hydroxylation pattern of the two aromatic rings demands different building stones. Much more information concerning the biosynthesis of aromatic compounds in general is needed before the origin and nature of the flavonoid precursors are revealed.

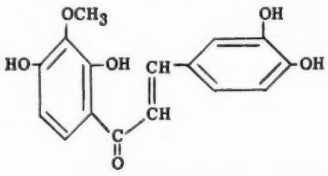
In some cases glycoside formation seems to be the last step of biosynthesis. In maturing flowers of *Coreopsis grandiflora* the content of free butein diminishes and that of butein glycoside increases during the 10 days of flowering (1a). In a yellow variety of *Dahlia* the main glycosides of various aglycones carry a glucose unit at corresponding hydroxyls which is in agreement with the concept of glycosidation as being the last step (56). However, both the stage at which glycosidation takes place and its possible importance in directing the flavonoid synthesis, are at present unknown.

Since the production of one pair of flavonoid compounds in the *Dahlia* can be suppressed by the action of genes which are responsible for the production of other pairs, the different compounds stem from a common source. These resources are probably limited in quantity and competition occurs between the types which are possible according to the genetic complex.

The flower colours of other species belonging to the Heliantheae tribe of the Compositae have been intensively studied. In *Coreopsis grandiflora* the yellow pigment has been identified as the leptosidin glycoside leptosin (21, 23). In two other *Coreopsis* species (*C. lanceolata* and *C. saxicola*), apart from leptosin and its aglycone, a new chalkone, lanceolin, has been discovered (82). The aglycone lanceoletin has essentially the same hydroxyl pattern as the aurone leptosidin. In *Cosmos* species flavones occur which fit into the sub-



Leptosidin



Lanceoletin

stitution pattern of both the co-occurring chalkones and the aurones. The side-by-side co-existence in these species of the glycosides of chalkones with the aurones suggests that, from genetic evidence, an enzymatic interconversion should be possible if the gene-enzyme theory holds true. In point of fact, homogenates of powders prepared from ray flowers of *Cosmos sulphureus* or *Coreopsis lanceolata* are able to convert chalkones into aurones at a rapid rate at pH 5–6. The conversion is affected in the state of glycosides (83). This is, so far, the first example of an enzymatic transformation of one type of flavonoids to another.

There is continuing argument regarding the question whether oxidative or reductive steps are the final reactions responsible for producing visible flavonoids from possible colourless precursors. Robinson, one of the participants in the dispute, states his opinion as follows:

> Inevitably the transformation of leuco-anthocyanidins into colouring matter of flavylium type must occur in nature occasionally but it does not follow that this route represents the standard mechanism. Analogously we do not wish to exclude even flavonol reduction in special cases; we think there is ample evidence that it is not the usual mechanism of phytosynthesis. We have consistently maintained that the anthoxanthins (=yellow flavonoid pigments) and anthocyanins are formed in the plant from a common progenitor by forking of the roads (70, 72).

This picture is supported by observations from various directions and may be taken as a valuable working hypothesis. Under these aspects leuco-anthocyanins, too, are considered to be one of the final types of flavonoids. Their wide distribution throughout higher plants does not suggest them to be only intermediates in anthocyanin metabolism. They are encountered in many white flowers which never change to red or blue (2). In certain cases their conversion into coloured flavonoids is highly probable. The bracts of the male flowers in certain banana varieties are paler, the younger they are. The youngest ones contain exclusively unmethylated leuco-anthocyanins, leucocyanidin and leuco-delphinidin. In the mature bracts, apart from cyanidin and delphinidin glycosides, methylated compounds such as peonidin and malvidin glycosides occur and the leuco-anthocyanins disappear (87). In the buds of *Petunia* species a flavonol accumulates which is converted into the blue pigment just before blossoming (90). Although, in many cases, an apparent occurrence of free anthocyanidins in petals has turned out to be an artefact, recent careful observations seem to indicate that in several species traces of free anthocyanidins exist at least temporarily. In the red flowers of *Camellia japonica* var. *hortensis* the unglycosidised cyanidin amounts to a high proportion of the pigment present (32). In one variety of *Pharbitis Nil* the sugar-free anthocyanidin of the flower buds is converted into the glycoside during the development of the flowers and the deepening of their colour. In the petals of a *Gossypium* species the aglycons are reported to occur temporarily free (88).

In spite of the subtle dependence of flavonoid formation on genetic fac-

tors, changing conditions in the cell itself and environmental influences can be decisive factors in the pigment outfit of a certain variety. This possibility is demonstrated by frequent observations of flowers changing colour during development or before withering. One of the best known examples are the flowers of *Hibiscus mutabilis* which normally change during one day from white through pink to deep red (41). In certain species, e.g., *Hydrangea, Cineraria, Delphinium consolida,* the production of anthocyanins is completely inhibited in the dark (50). The reddening of *Dianthus barbatus* flowers, which are white during development, is stimulated by illumination (19). Other similar cases have been reported [cf. (12)]. Although the favourable influence of illumination on anthocyanin formation has been frequently observed in other plant organs, too, light is by no means an indispensable factor of anthocyanin biogenesis. There is so far no definite explanation or suggestion of how light could interfere with the synthesis of flavonoids. According to experiments with green leaves the influence may be indirect as a result of an increased mobilisation of raw material through normal carbohydrate breakdown [cf. (17)]. In many species temperature during flower development markedly affects pigmentation. Investigations into general metabolism, particularly quantitative estimations of the production of flavonoids running parallel with estimations of carbohydrate breakdown, would provide more reliable information than is at present available on the influence of both light and temperature.

Inheritance of Flower Colours

The interest in flower colour genetics is very old and has grown from different sources. Mendel made great use of flower colour to follow the fundamental paths of gene action. Much of the plant breeders' success depends on the knowledge of genetic factors that control the flower colours of a great number of garden varieties of plant species. Pioneering work in biochemical genetics was done with flower colours long before the "classical" work with microorganisms was started [cf. (44, 77)]. Modern chromatographic techniques are introduced into this field (94).

Numerous genetic factors are involved in the process by which pigments are produced from their raw material, supposedly carbohydrate broken down in the general glycolytic pathway (10, 11, 17, 18, 62). Other genetic factors have been detected which modify the pigment, produce co-pigments or change the cell conditions in such a way as to produce a wide range of tones from one type of pigment. Plastid pigments are genetically independent of flavonoids (16) and do not interact chemically with them unless the supply of raw material is concerned. The presence or absence of a carotenoid pigment in *Cheiranthus cheiri* is governed by a gene Y. In flowers also containing anthocyanin the yellow pigment has a strong background effect and the flower colour is brown as compared with the purple or pink of the plants recessive for Y (76a). In *Eschscholtzia californica* one factor is responsible for the production of carotenoids in a low concentration. Other factors deter-

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mine which carotenoids are produced, mainly esters of various xanthophylls. Moreover, the area of the petals covered with pigments is genetically determined and an intensification factor working to deepen the colour obviously makes a common building stone more readily available (16). *Eschscholtzia* flowers contain flavonoid pigments as well. Their production and relations are substantially the same as shown below for *Dahlia*. A common precursor for anthocyanins and flavones seems to be directed by genetic factors into specific channels. More genetic experiments which could well provide a starting point for the elucidation of carotenoid genetics in flowers have been carried out with the carotenoid pigments in tomato fruits by Porter & Lincoln (63) and Mackinney & Jenkins (51).

The close relationship between single genes and simple biochemical differences was first demonstrated with flower colours as early as 40 years ago (59, 60). One of the main results achieved in this way was the disproving of the theory that a simple relationship existed between the structure of anthocyanin and flavone in pigments in *Antirrhinum majus* flowers. Here cyanidin and apigenin are present whereas apigenin would be related structurally to pelargonidin (76). In *Lathyrus odoratus* the flavonols in highly pigmented varieties were the same: quercetin and kaempferol, no matter whether the anthocyanin accompanying them was of the pelargonidin, or cyanidin type (8). Leuco-anthocyanins in the seed coats, however, show a correlated variation with the anthocyanins in flowers. Most cases of correspondence are confined to the 3′,4′-dihydroxy substitution represented by quercetin and cyanidin. A lack of correspondence is the rule (22).

General considerations of genetic phenomena: mutation, dominance and epistacy, led to the conclusion that cyanidin derivatives are synthetically simpler then delphinidin although the relation of pelargonidin derivatives to the other anthocyanidins is not clear (7). Cyanidin and quercetin are the most widely distributed of their groups. Palargonidin and kaempferol or delphinidin and myricetin require an extra step in the respective removal or attachment of a hydroxyl group.

Numerous genetic observations demonstrate that various types of flavonoids are synthesised from a common precursor which is probably limited quantitatively (see p. 291). If much is used in the production of one member, less is left for the action of genes producing other types. In *Papaver rhoeas* competition for the common source is in favour of the anthocyanins at the expense of the flavone. In this and other cases, a co-production is most likely due to parallel rather than sequential synthesis (8). In certain genetic combinations of *Gossypium* a colourless substance of yet unknown composition is formed in the petals which may be the common mother compound for both cyanidin and quercetin (88, 89). In *Lathyrus odoratus*, dominant alleles C and R are responsible for the production of anthocyanins at the expense of yellow flavonoids which appear when the recessive alleles are present. In *Antirrhinum* and other species recessive white varieties exist in which the recessive alleles allow no production of either red or yellow

flavonoids. The synthesis in these genotypes is obviously blocked at a very early period. No attempt has so far been made to isolate a definite compound presumed to be such a common precursor [cf. (8)].

Some of the general rules for flower colour heredity can be demonstrated with the extensively studied genetic composition of *Dahlia* garden varieties which are autotetraploids. This, in some respects, complicates the genetic analysis because of the combinations of the tetrameric alleles. On the other hand, it provides the possibility of studying competition and suppression effects. In *Dahlia variabilis* four main factors govern flower colours; two for anthocyanin and two for flavone production. Their specificity is as follows: A, light anthocyanin pigmentation either by canidin or pelargonidin; B, heavy anthocyanin production either by cyanidin or pelargonidin; I, ivory flavonoid; Y, yellow flavonoid; a fifth factor which, in the polyploid varieties, is tetrasomic, too, progressively inhibits the formation of yellow flavone giving rise to the intermediate cream and primrose colours (45). Pronounced interaction between the different flower colour factors can result in partial suppression of the pigments, depending on the total number and competition value of the alleles present which govern the synthetic processes. Generally, the flavones suppress the anthocyanins more strongly than vice versa, e.g., Y and I together cause complete suppression even of B (heavy anthocyanin formation). The action of I on B results in the depression of B so that only light formation of anthocyanin takes place.

What does this mean when translated into biochemical terms? Any given stage in a series of reactions can be achieved only if a number of conditions are favourable. These different steps are controlled by different genes; even small changes frequently need several genes. In *Lathyrus odoratus* a flavonoid co-pigment is produced only when two genes are combined (48). If one of them is lacking the whole reaction is stopped. It must not be assumed that the unifactorial difference between a genotype producing pelargonidin and one producing cyanidin means that only one gene is required for the formation of either substance. The pelargonidin type may already contain all except one of the factors necessary for the formation of cyanidin (7).

This general picture, however, does not hold true for the circumstances allowing competition between various flavonoids. In these cases suppression of one substance leads to the production in increased quantities of another compound which is derived from the same raw material as the first. In such cases the main line of synthesis of the dominating substance has already been laid down in the original genotype. Biochemically, suppression may be brought about by the new gene combination changing the reaction velocity. Alternatively, the enzymes which direct the common building stones into one or the other pathway may be produced in increasing or decreasing quantities. Such quantitative relationship is supported by the particular composition of the *Dahlia* flavonoids given above (see p. 286) where a series of flavonoid compounds with related features are present in the same flower type.

Apart from genes which are responsible for the production of the pigments

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themselves there are many other genes controlling the localisation and pattern of the pigments. Moreover, genes have been found which convert the acidity of the cell sap, a factor which plays a certain role in the modification of flavonoid colours. Such genes have been shown to act in *Primula sinensis*, *P. acaulis*, *Papaver rhoeas*, *Tropaeolum majus*, *Lathyrus odoratus*, and *Trifolium pratense* [cf. (48)]. These observations would suggest that minute changes of pH values have a more important influence on the colour tones of flowers than were found by other investigations which will be discussed below. There has been no attempt to find out the biochemical mechanism producing a more or less acid cell sap.

One case may be quoted at some length to illustrate the results of the independent segregation of various genes which act on the oxidation and methylation of the anthocyanidin molecule, on the production of co-pigments, and on the acidity of the cell sap (47, 48). A wide range of flower colours can be produced from the combination of only a few genes. Three of the major gene pairs governing flower colours in *Primula sinensis* are Kk, Bb, and Rr. K gives rise to malvidin-3-galactoside and k to the corresponding pelargonidin glycoside. B produces a flavonoid co-pigment and R increases the acidity of the cell sap. On self-fertilisation of a plant heterozygous for all 3 genes the following classes and ratios are obtained.

27 KBR magenta: co-pigmented, malvidin glycoside, low pH
9 KbR red: unco-pigmented, malvidin glycoside, low pH
9 KBr blue: co-pigmented, malvidin glycoside, higher pH
3 Kbr slaty: unco-pigmented, malvidin glycoside, higher pH,
9 kBR almost white: pelargonidin plus yellow flavonoid, low pH
3 kbR coral: pelargonidin glycoside, no yellow flavonoid, low pH,
3 kBr almost white: pelargonidin glycoside plus yellow flavonoid, higher pH
1 kbr pale coral: pelargonidin glycoside, no yellow flavonoid, higher pH

Based on experimental data collected from a wide variety of plant species, certain general rules can be deduced since a great similarity of behaviour as to dominance and interaction of the genetical factors concerned with flower colours has been found (76a).

(*a*) Plastid pigment, co-pigment, and yellow flavonoid together with both general and specific anthocyanin production are generally dominant over their absence. (*b*) The modification involving more oxidised anthocyanins is dominant over less oxidised and the more methylated is dominant over less methylated. (*c*) 3,5-diglycosidic and complex (acylated) anthocyanins are dominant over the 3-monoglycosidic and normal anthocyanin type. (*d*) More acid petal sap is dominant over less acid. (*e*) Uniform pigment distribution is generally dominant over flaking or marbling.

In various *Primula* species the aglycon of the anthocyanin pigments is identical (malvidin) whereas the various pigments differ in the nature and position of the sugar residue (76).

The inheritance of *Verbena* flower colours is contrary to the rules for other plants in the following respects (9): (*a*) Pelargonidin derivatives are

sometimes dominant, sometimes recessive, to delphinidin. (*b*) Monosides are sometimes dominant, sometimes recessive, to di-monosides. (*c*) Mixtures of anthocyanins occur either because of incomplete dominance or because of modifying factors. Here again, a better insight would be obtained if not only the substances but also the process producing the final compound were investigated.

A unique change of dominance with age in flowers has been reported with hybrids of two *Aloe* species, one having red flowers and the other yellow. All F_1 plants develop flowers which are red in the bud but yellow after the bud has opened (65).

Summing up, we may say that generally we know what the gene is doing although we do not know how it does it. Some of the central problems arising from this situation have been briefly outlined by Haldane (30):

A. A search for enzymes responsible for biochemical differences, e.g., pelargonidin oxidase, and for inhibitors of enzymes. Thus, one would look for the absence of an enzyme in a recessive white, for the presence of an inhibitor in a dominant white.

B. A full investigation of the sugars of anthocyanins and other flavonoids. Are they correlated with other glycosides or with carbohydrate stores in the same plant?

C. Further work on methylation and an attempt to link it up with the metabolism of methionine or betaines.

D. Investigation of the origin of pH differences (with simultaneous work on the same question in fruits where it is of economic importance).

E. Attempts to alter lower colour by injecting (*a*) a possible selective enzyme inhibitor and (*b*) possible missing metabolites.

Some of the problems may not have been put in terms suitable for immediate approach but nevertheless they point to questions which can be approached with modern methods. It may be added that the frequent occurrence of rhamnose as sugar component of various flavonoid glycosides can not yet be explained by any metabolic relation.

Colour Tones and Patterns

The actual colours displayed by flowers are generally not the colours of pure pigments either of the flavonoid or carotenoid type. The colour of a flower depends first upon the pigments present and, secondly, upon modifying conditions such as the acidity or other components of the cell sap. The plastid pigments combine with the water-soluble ones to give the tone and pattern of flower colour either by mixture or by background effects.

Plastids may be absent, as in the red rose; present in a central "eye" as in the primrose, or present throughout the petals as in many Cruciferae [cf. (30)]. Most brown flowers contain anthocyanin in addition to carotenoids, e.g., garden varieties of the wall flower, *Cheiranthus cheiri*. The scarlet colour of many tulip varieties is the result of a mixture of cyanidin derivatives and carotenoids. *Tropaeolum majus* flowers contain little cyanidin glucoside and plentiful carotenoids (98). When yellow flavonoids are present together with

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carotenoids the actual colour is normally determined by the carotenoid pigment even if it is quantitatively not predominant. Carotenoids generally impart much heavier colours to the petals than yellow flavonoids. In yellow *Viola tricolor* varieties it is not the large quantity of quercetin but the small amount of violaxanthin, a carotenoid, which imparts the yellow colour to the petals. The deep yellow colour of *Forsythia* flowers is mainly caused by plastid carotenoids. The flavonoids present contribute but slightly to the visible colour [cf. (81)].

Many polyhydroxyflavones are themselves only weakly coloured. However, their salts are deep yellow. Thus, when a flower which contains either uncoloured or pale yellow flavonoids is exposed to ammonia vapour it turns deep yellow. There are very few examples known in which the white petals do not contain some flavonoid compound. In recessive white *Dahlia* forms the flavanones naringenin and eriodictyol are present (56). The bees may react differently to an object coloured by a "white" flavonoid which absorbs ultraviolet light than to one, such as white paper, which does not. Instructive photographs have been given to show the colour effects of ultraviolet light absorption by flavonols in flowers (29).

When other conditions are identical, the pure anthocyanins have different tones dependent on their chemical structure (cf. p. 274). In *Verbena* hybrids are four colour variations which demonstrate very clearly the single and combined bluing effect caused independently by the two separate modifications of the pigment molecule, i.e., hydroxylation and change in glycosidal type (77). In a population of dark purple cornflowers containing cyanidin derivatives a certain number of pink coloured individuals appeared which produced pelargonidin (97).

The following factors which can modify the tone of a certain anthocyanin have been singled out: (*a*) the concentration of the pigment in the cell sap; (*b*) the acidity of the cell sap; (*c*) the kind and concentration of inorganic ions in the vacuole; (*d*) the presence of so-called co-pigments; and (*e*) colloidal components of the cell sap which may effect a stabilisation of anthocyanin salts.

The "pigment concentration" can vary in a rather wide range. In the common cornflower the petals contain about 0.65 to 0.70 per cent cyanin as calculated on dry weight. A dark purple garden variety accumulated up to 14 per cent cyanin. *Pelargonium peltatum* flowers contain 1 per cent, *P. zonale* 6 to 14 per cent pelargonin in the dry petals (97). The concentration of violanin in dark violet *Viola* varieties is reported to be as high as 30 per cent of the dry weight. In certain flowers the anthocyanins are even precipitated in the vacuoles [cf. (42)].

It appears that the importance of the role of the much-discussed "acidity factor" has been overemphasised in the colour-determining and colour-changing process to which it is linked. *In vitro* it is easy to produce a wide range of tones between red and blue with almost every anthocyanin by variation of the acidity. In the petals the colour which is displayed by a certain anthocyanin does not simply depend on the acidity of the cell sap in which

the pigment occurs. Cyanin is blue in the acid cell sap of corn flowers at a pH value of about 4.8. The same anthocyanin is responsible for the colour of the red rose with approximately the same pH value in the cells. It is concluded that the corn flower contains three modifications of cyanin, namely, the purple form of cyanin itself, the blue form which is its sodium salt, and the red oxonium salt of cyanin with some organic acid present (69, 95, 96). A pH value above 7 is very rare in plant cells and most of the deep blue tones are not dependent on an alkaline reaction of the cell sap. An examination of 169 species containing anthocyanins in flowers or fruits revealed that the pH value was always below 7, irrespective of the colours and that the pH value varied in the same range whether the flowers were red, violet, or blue (33).

The blue *Hydrangea hortensis* varieties owe their colour to an acid stable aluminum-delphinidin complex. However, it was not possible to produce a colour change towards deeper blue under experimental conditions in several species which contain delphinidin derivatives like *Hydrangea*. In some other species, e.g., in *Stuartia* the delphinidin-aluminum compound is responsible for a deep blue colour (14). Acid-stable, cyanidin-aluminum compounds also exist. No simple relationship between total ash content and flower colour has been found when a great number of blue and red flowering plants were compared (52, 85). However, the alkalinity of the ash, i.e., the excess of alkaline components over the acid compounds is generally greater in the blue flowering species than in related red flowers (52). Referring to the separation of a stable blue pigment from petal extracts by precipitation with alcohol, Shibata & Hayashi (84) point out that this blue pigment always contains a considerable amount of alkaline ash substances such as Mg and Ca. The red pigment could not be precipitated in the same way but when Mg or Ca salts had been added to a red extract of anthocyanins a stable blue compound with essentially the same properties as the natural blue substance was formed. The authors, therefore, conclude that anthocyanins are capable of forming metal complexes with various ions such as $Mg^{\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$, which are rather stable and which remain blue even in acid solutions [cf. also (31)]. The precise structure of these anthocyanin metal complexes must be revealed by further chemical analysis.

Co-pigmentation, a very important factor in the determination of flower colours, was first investigated by Robinson & Robinson (66). In the extracts of fresh petals, substances (co-pigments) exist which intensify or modify the colour of the anthocyanin present although the substances concerned may themselves have a very pale colour or none at all. Co-pigments probably exist for all types of anthocyanins although very few have been identified so far. Co-pigments with slight blueing effects on oenin solutions are tyrosin, arbutin, catechin, etc. Aesculin and tannin have a strong effect. "The phenomenon has little or nothing to do with salt formation. It is evidently the result of formation of weak complexes which are dissociated at an elevated temperature or by the action of solvents (66)." It is obvious that a genetic factor for flower colour change may be concerned only with the development

of a co-pigment, the anthocyanin remaining unchanged in composition and concentration. This is the more likely in many cases where, apart from anthocyanins, other flavonoids are present in the same petals which generally have a conspicuous co-pigment activity.

The deep blue colour of the corn flower and of other blue flowers can not be explained by any of the mechanisms mentioned so far. In the cell sap of the corn flower colloidal substances such as xylans and other polysaccharides have been found. These colloids seem to stabilize the blue pigment anion in spite of the high acidity of the cell sap (67, 68, 72).

The origin of colour patterns on petals is still more complicated than that of the natural colour tones. Important contributions to the first experimental approach to colour patterns in flowers have been made by Harder and coworkers, (29), Vogel (93), and others. Troll (92) has given extensive descriptions of the underlying problems. Only very few general aspects and conclusions have so far emerged in this field. There are certain areas of the single petal and the flower tube which preferably develop colours different from the main flower colour. Such parts are, e.g., the base of the petal and the central "eye" of tubular flowers.

Various flowers normally characterised by spots and speckles lose or change this pattern when grown under unusual light or temperature conditions. *Calceolaria hybrida grandiflora* possesses spotted flowers. When cultivated at 30°C. the flowers progressively produce less anthocyanin spots and become uniformly yellow. At the same time they become smaller as judged by the photographs which seem to indicate a general starvation or exhaustion in metabolism at those higher temperatures. In *Petunia* flowers during development, the violet pigmented areas increase as the temperature is lowered. Moreover, they were less speckled as the light intensity was increased during an early phase of flower development [cf. (29)]. The temperature and light treatment must be given when the flowers are still in bud. However, the production of anthocyanin does not begin before the petals have reached a considerable length. In this phase of development light and temperature influence the amount of anthocyanin produced but almost in an opposite direction. Low temperature combined with high light intensity during the bud stage reduces the coloured area but during the further development results in high pigment concentration and, therefore, gives deep blue spots.

The white areas of *Petunia* flowers do not contain any flavonols or leucoanthocyanins. Genetically, the production of anthocyanins in *Petunia* is governed by one single gene, the absence of which leads to the production of a flavanol whose distribution pattern is the same as that of the anthocyanin (90). The genes responsible for this pattern are independent of those which govern the type of flavonoid produced. The genetic interpretation of variegated flowers, i.e., speckled or striped petals shows that this phenomenon is either due to the pattern manifested by certain genes or it is caused by mutable genes with or without the co-operation of labile plasm. In the first case, variegation is generally independent of genetic changes of the background colour (35).

Although no generalization is allowed as to the cause and development of colour patterns, the *Petunia* case may indicate that the extent of coloured area on a flower is not simply a question of anthocyanin production. The origin of speckles, stripes, and other designs must be connected with other phenomena of plant development such as pattern of stomata, sklereids, and other idioblasts, whereas the amount of pigment produced in a certain area is mainly the result of an abundant or a starving metabolism. Here, again, a better knowledge of the chemical pathway of biogenesis of flavonoid and carotenoid pigments would help greatly to prepare the way for solving the many problems of the origin of colour patterns in flowers, a field in which a close co-operation is essential between geneticists and plant physiologists concerned with development and metabolism.

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PHYSIOLOGY OF THE COTTON PLANT[1]

By Frank M. Eaton

Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture, College Station, Texas

Introduction

A measure of distinction is attached to the physiology of the cotton plant by reason of the dimorphism of its branches, its indeterminate growth habit, and the shedding of small floral buds and young bolls. According to environmental conditions, the branches produced by the cotton plant may be either vegetative or fruiting; the Upland cottons of the South produce variable proportions of the two. Customarily, one or more vegetative branches are developed from the lower sixth to ninth main-stalk nodes after which fruiting branches appear at the successively higher nodes. The interval between the appearance of new fruiting branches is about three days and between the successive nodes of the fruiting branches (each with a floral bud) there is an interval of about six days. Flowering is thus progressive and for a time it becomes more rapid as the plant grows. After flowering and boll setting have continued for a number of weeks, however, both the growth of the plant and the production of flowers are checked and may stop. Also, many, if not most, of the floral buds abort before anthesis. The flowering and fruiting behavior of American Upland cotton, *Gossypium hirsutum* L., is day-length neutral; however, there are short-day cottons which develop only vegetative branches during long days. Without the advantage of suitably high temperatures, the day-length neutral cottons behave like short-day cottons under long days and produce only vegetative branches.

Structure and Development of the Cotton Plant

The following summary of growth habit is stated in terms of the usual pattern in American Upland cottons. The statements are drawn extensively from Cook & Meade (1) , Cook (2, 3), Hayward (4), and Gore (5). The main stem of the plant has an indeterminate growing point that produces leaves in a three-eighths spiral. Two buds are formed at the base of each leaf. If the axillary bud develops it produces a vegetative branch. If the extra-axillary bud, which may be located either to the right or left of the axillary, develops it produces a fruiting branch.

The vegetative branches are structurally like the main stem and may replace the main stem. In addition to arising from the main stem of the plant, vegetative branches may develop from any of the axillary buds of either the vegetative or the fruiting branches. More vegetative branches are developed by wide-spaced than by close-spaced cotton plants, and more are developed

[1] The survey of the literature pertaining to this review was concluded in June, 1954.

under humid (2) than under dry conditions. With the systematic removal of floral buds the plant develops large numbers of vegetative branches and the development of these is stimulated as well by removing the terminal bud and by partial defoliation. A stimulated development of vegetative branches can be brought about, also, by covering the top several nodes and leaves of a plant with a black cap (6). Cool nights (7), in the absence of high-day temperatures, result in plants composed of vegetative branches and with only a few rudimentary fruiting branches. This reaction is also displayed by short-day cottons when grown under long-day conditions.

The structural differentiation between vegetative and fruiting branches, together with the reactions of Upland cottons to low temperatures and of short-day cottons to long days, has led the writer to regard fruiting branches as being homologous to inflorescences. Among other plants dimorphic branches are seemingly quite unusual (2) but it is not unusual in many species for blooms or inflorescences to be developed from extra-axillary buds.

As the fruiting branch grows from the mainstalk or from a vegetative branch, it is terminated by the meristem that produces the next floral bud and the extension of the shoot. The floral bud starts to develop first and is then pushed aside by the new shoot to a position almost opposite the petiole of the leaf. The new shoot also comes out at an angle. Cook & Meade (1) have pointed to several reasons why the fruiting branches of cotton are not true sympodia (see Hayward also), but the jointed zig-zag appearance of the fruiting branches has sometimes led to their being termed sympodia. Although the lowest fruiting branch ordinarily appears at the seventh to tenth mainstalk node in Upland cottons, it has been noted (1) in some instances as low as a third or fourth.

In the growth and development of the cotton plant there is, according to temperature, usually a variable interval of five or eight weeks, or longer in the case of short-day cottons under long days, after planting before the first square (floral bud) is visible on the first fruiting branch. In one set of observations (8) the first floral bud appeared 58 days after planting when the seeds were planted in Texas on April 2, and after 19 days when planted on either July 24 or August 15. About 23 days elapse before a newly-visible bud opens as a flower in American Upland cottons and 30 days in Egyptian cottons. According to climatic conditions and variety it requires an additional 40 to 60 days, or longer, for a flower to develop into a mature boll. Open bolls are usually not expected to appear in much less than four months after planting. With increases in mean daily temperatures, possibly together with increased transpiration rates, substantial reductions have been noted in boll periods, in number of seeds per boll, and in the weights of seed and lint per seed. On the basis of scattered data, the amount of seed cotton per boll may be reduced as much as 15 per cent, or more, in response to a 10°F. higher mean daily temperature. An extensive literature on bud and boll periods and growth conditions that affect these periods has been reviewed by Ludwig (9).

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Reeves & Beasley (10) show the cotton seed embryo to have a dry weight of 3 mg. 18 days after fertilization and to contain at that time oil, starch, pentosans, gossypol, and proteins; glucose could not be identified. According to a number of investigations [see Anderson & Kerr (11)] the lint cell reaches its final length during the first 15 to 20 days after anthesis while enclosed in an extremely thin primary wall. Secondary thickening, through the deposition of cellulose on the inner surface of the primary wall, then continues until the final dry weight of the boll has been attained; by this time the volume of the lumen is greatly reduced. The gain in dry weight of the boll is slow during the first five or six days after anthesis and then tends to be linear (6) but a period of more rapid than average gain in weight centered at about 15 days. In the measurements reported on Acala cotton in Arizona the final boll diameter was reached in 15 days; these bolls stopped gaining in weight at about 38 days and opened in 45 days.

Germination and Seedling Growth

To a greater degree than applies to many crop plants, the germination of cotton seed is favored by high oxygen, high moisture and high temperature. The calcium requirement also appears to be high. Long ago, the Egyptian fellahin learned to cover their cotton seed with sand, the seed being placed in holes pressed out with dibbles on the warm south bank of irrigation ridges.

Hutchins (12) has shown a higher oxygen requirement for the germination of cotton seed than for the seed of maize, wheat, and rice. This finding is plausible when it is noted that the cotton seed kernel, compared to maize, contains on a percentage basis eight times more oil and five times more protein. The oxygen requirement for the conversion of these materials to more labile compounds is such that repeated replanting is often necessary when rains seal the soil against aeration before germination occurs.

The cotyledons in a cotton seed are relatively massive and their hydration requires a large intake of moisture to prepare them for subsequent photosynthetic activity. But, as shown by Stiles (13), the embryo (excluding cotyledons) after 96 hr. contains almost the same moisture (approximately 1100 per cent) as the embryo of maize. Many cotton planting machines have rubber press wheels that compact the cotton seed against the moist furrow bottoms to facilitate water up-take but the seeds are covered with loose soil.

At the same locations cotton is planted a month or more later than maize; currently, tests are being made to determine the soil temperature at a depth of 8 in. which will provide some assurance of good cotton germination. Arndt (14) has studied the temperature relations for the growth of cotton hypocotyls and roots and compared his results with the literature on wheat and maize seedlings. The optimums for cotton, maize, and wheat are given as 33–36°; 32° and 20–25°C., respectively; the respective minimums were 18°, 12°, and 10°C. Arndt germinated and grew his seedlings on mineral nutrient agar in large test tubes in the dark. The most rapid growth of primary roots and hypocotyls occurred at 33°C. but the results were nearly the same at

36°C. At temperatures of 30°C. or above the secondary roots appeared on the fourth day. Respiratory loss in organic matter during the first seven days was rather uniform between 24 and 33°C. but a sharp increase was shown at 36°C. In agreement with Camp & Walker (15) germination was not successful at either 14 or 40°C. Under field conditions, germinating cotton seed and their hypocotyls are particularly subject to fungal diseases; this accentuates the need for rapid seedling growth.

Presley & Leonard (16) have found Upland cotton seedlings to be more sensitive to calcium deficiency than were any of the eight species with which they were compared. The radicles were injured by distilled water, sodium bicarbonate-type tap water and, when added singly, by salts of magnesium, potassium, and sodium.

After germination, the cotyledons are carried well above the soil by the elongating hypocotyl. They then double their area and synthesize new carbohydrates for a period of 10 or 12 days before the first true leaf develops and enlarges to the size of the cotyledons. During this early growth period, and before the shoot starts to elongate, the primary root extends downward into the soil to a depth of 20 cm., or more, and many lateral roots are formed. The activity of these early roots in nitrogen accumulation is indicated by Crowther's (17) rather surprising but significant correlation between the nitrogen in the cotyledons (3 days after emergence and 7 days after planting) and the final yields of Egyptian cotton in the Sudan. In this between-years correlation, soil rather than seed nitrogen was believed to be represented.

Moisture Relations

Water Requirements and Leaf Temperatures.—The mean water requirement of Upland cotton as measured by Briggs & Shantz [see (18)] at Akron, Colorado, was 568 (ratio of water absorbed to dry matter harvested); this value was substantially higher than their value for maize (350) but was substantially lower than the value for alfalfa (844). Under the more arid conditions of Arizona, King (19), on the basis of field measurements by soil sampling, obtained a water requirement value for Pima Egyptian cotton of 874 as the mean of two water-supply treatments. Eaton & Belden (20), using Briggs and Shantz equipment and methods with the consequent greater exposure of the plants, at Sacaton, Arizona, obtained values of 891 and 1042 for Acala Upland and Pima Egyptian, respectively. In Arizona, Hawkins (21) has shown the leaves of Pima cotton to contain less moisture and to desiccate more during the day than Acala cotton; a difference of 2 percentage points in the early morning became 5 per cent in the late afternoon.

The leaf temperatures (20) of turgid Pima cotton are lower than those of Acala which indicate higher transpiration rates for the former; as the mean of 10 series of measurements the difference was 2.4°C. Possibly associated with the higher leaf temperatures of Acala Upland was the observation that the mean yield of Acala cotton in Arizona had been 40 per cent greater for three

summers with average daily maximum temperatures below 100°F., than for the three alternate summers when the temperatures averaged above 100°F. There was no apparent relation between air temperatures and the yields of Pima Egyptian cotton. Wilted leaves of cotton plants with transpiration rates below ten per cent of turgid plants had temperatures 2°C. above the air at a time when the turgid leaves were 3.5°C. below air temperatures. Balls in Egypt (22) also found a 5°C. difference in the temperature of turgid and wilted Egyptian cotton leaves. The thermal death temperatures as observed in young cotton plants by Berkley & Berkley (23) for 30 min. exposures were in the order of 52°C. at high humidity and 59°C. at a lower humidity. Further illustrating the effect of transpiration rate on tissue temperatures, was the fact that cotyledons survived at temperatures which killed the hypocotyls.

Using mainstalk leaves of 20 to 82 day-old Pima Egyptian cotton plants grown without branches, Eaton (24) found, in 1927, that the cell sap concentrations increased and transpiration rates (measured by leaf temperatures) decreased with leaf age. But leaves younger than 20 days had both low sap concentrations and low transpiration rates. During the 22 days after leaf removal, 9.9 new terminal leaves were formed by each of the defoliated plants but only 5.9 by plants that had not been defoliated. The new leaves of the defoliated plants were over three times as large as the control leaves. In the discussion of the regrowth, consideration was given both to the reduction in moisture tensions by defoliation and to a possible growth-inhibiting substance transmitted from the old leaves to the young.

Root development, Growth and Fruitfulness.—The relation of water supply to the distribution of growth between various parts of the Upland cotton plant was measured by Eaton (25) at Sacaton, Arizona. Plants grown in Briggs and Shantz type water-requirement cans were: (*a*) kept turgid and, (*b*) allowed to wilt before each addition of water. The effect of the treatments on the dry-weight distributions as percentages of the total weights at the end of the season were as follows: leaves plus stems, wet 37 versus dry 39 per cent; buds plus bolls, wet 49 versus dry 49 per cent; roots, wet 14 versus dry 12 per cent. The total dry plant weights (grams) under the two treatments were: wet 541 versus dry 326 gm. The foregoing data leave little basis for concluding that a deficient water supply has a significant effect either on relative fruitfulness (bolls per unit of vegetative weight), or that it stimulates root development. In other words, water supply did not alter the growth partitions.

King (19) has reviewed the early literature on the relations of water supply to boll shedding. In his irrigated plots of Egyptian cotton in Arizona he found smaller percentages of bolls shed as the frequency of irrigation was reduced but there were reductions in yield. Roots were traced to depths of almost 11 ft. Percentages of bolls shed by Acala cotton as observed by Beckett & Dunshee (26) were not materially influenced by the frequency of irrigation at Shafter, California. With consumptive uses of 39, 25, and 23 acre-inches

of water, they obtained estimated yields of 3100, 2300, and 1810 lb. of seed cotton per acre. In a later investigation, Adams, Veihmeyer & Brown (27) found increased vegetative growth but not increased yields with heavier irrigation schedules. At the time of these latter investigations, the destructiveness of Lygus bugs to squares and young bolls had become prominent in the San Joaquin Valley and there were no adequate means for control. This insect migrates from dry to irrigated cotton and it seems possible, if not probable, that the failure to obtain increased yields with increased water in these studies can be accounted for on this basis.

The cotton plant may wilt severely and yet show a good recovery following rains or an irrigation. The hour of wilting is often used as a guide to irrigation. Marked reductions in growth have also been found to occur without wilting. Acala and Stoneville cottons grown by Eaton & Ergle (28) at Shafter, California, were irrigated weekly on both sides of each row and on one side biweekly. The latter plants never wilted but the late afternoon phototropic responses were reduced. The fresh weights of leaves and stems of the two varieties were lowered, respectively, by 53 and 50 per cent by the limited water supply and the number of bolls by 44 and 46 per cent; the relative fruitfulness of the two varieties was increased, respectively, by 20 and 4 per cent under the deficient water supply, but the bolls were smaller. In a similar experiment the following year with another variety on a lighter soil there was much wilting but the reduction in weight of leaves and stems was also about 50 per cent. The rather common view that drouth causes an unusual proportion of bolls to shed may originate in part from the fact that late summer drouth often coincides with the period of heavy boll shedding.

Anderson & Kerr (29) have found the enlargement of young bolls to be uninhibited by severe wilting of the parent plant, but after the boll cells had attained full size (about the time that secondary thickening of the lint cells starts) shrinking occurred during the day with recovery during the night; provided low soil moisture was not a limiting factor.

Wilting and Transpiration in Response to Low Substrate Temperatures.—Rather striking relations between root temperatures and water uptake have been developed by Arndt (30). Depending on factors influencing transpiration rates, wilting of the cotton plants became pronounced in several tests when the culture-solution temperature was dropped to 18.5°C.; with higher transpiration, plants wilted at 20° and 21° and in other tests at high humidity only at temperatures as low as 10°. Arndt suggested that increased viscosity of the protoplasm was among the factors which may have been responsible for the sharp reductions in water movement through the roots; he noted that sunflowers behave similarly to cotton in their low temperature-wilting relation. In another experimental approach to this subject, Kramer (31) found approximately a two-fold increase in the transpiration rate of cotton plants as the soil temperature was increased from 12° to 15°C. In contrast to the more linear soil temperature-transpiration relation found for some

other plants, the relation for cotton was sharply sigmoid. In areas characterized by cold nights, the morning wilting of young stands of cotton sometimes raises a question in the minds of cotton farmers on the need of replanting.

Moisture and Carbohydrate Accumulation.—In cotton leaves as studied by Eaton & Ergle (32) drought has been observed to cause significant increases in hexose sugars, variable but minor effects on sucrose, and sharp decreases in starch. In the stems and roots, on the other hand, there were always moderate to large increases in hexose, sucrose, and starch. It seems evident that water deficit does not impede translocation. On the basis of averages of leaves, stems, and large roots, the carbohydrates in the plant as a whole were doubled by a protracted drought. Drought thus appears to depress carbohydrate utilization by the cotton plant to a greater extent than it does photosynthesis. A review of the literature indicated that this is not always the case among other plants. Earlier data by Dunlap (33), in which the various carbohydrate fractions were not differentiated, were not always in agreement with the foregoing.

Salt and Water Relations

Wadleigh & Gauch (34) regard osmotic forces of the substrate as having the same effect in limiting water uptake as do the soil moisture tensions resulting from the drying of the soil. Adding the osmotic pressures of saline substrates to soil moisture tensions and expressing the sum in atmospheres as the "integrated soil moisture stress" they found cotton leaves to cease to elongate as the total force approached 15 atm., but at this force water was still being supplied for transpiration; no mention is made of wilting.

When cotton was grown, together with five other crops, by the writer (35) in large outdoor sand cultures differentially supplied with chloride and sulfate salts (in each case as 50 per cent sodium) the yield of seed cotton was reduced by 39 per cent by 100 m.eq./l. of chloride (mean of cultures with 50 and 150 m.eq./l.) and by 43 per cent by 200 m.eq./l. of sulfate (mean of cultures with 150 and 250 m.eq./l.). In this experiment the ratio of dry weight of seed cotton to dry weight of leaves, stems, and roots was 0.43 when on the control nutrient, a mean of 0.46 was found for the two chloride solutions, and one of 0.59 for the two sulfate solutions. The data indicate that although production is markedly reduced by saline substrates relative fruitfulness may be increased slightly. It will be noted later that both under low and high light intensities, nitrate concentrations high enough to retard growth (64 m.eq./l.) also increased relative fruitfulness.

Chloride concentrations found in the expressed sap from the leaves were increased from 18 m.eq./l. on the control substrate to 178 on the 150 m.eq./l. chloride substrate. Total sulfur, expressed as SO_4, was increased from 183 m.eq./l. on the control to 439 on the 250 m.eq./l. sulfate substrate. With the foregoing ionic increases there were increases also in the osmotic concen-

trations of the expressed sap; these tended to parallel the increases in the concentrations of the substrates. In some plants on some saline substrates the gains in sap concentrations exceeded the increases in the substrate and in other cases they fell below [(35) Table 8]. For all plants, in going from nonsaline to the various saline substrates, the average differential remained at about 11 atm. In those instances where the increases in sap and substrate concentrations were just equal, i.e., when the osmotic differential between sap and substrate remained unchanged, it has seemed to the writer that the net effect of salt in the soil on water uptake and its movement to the leaves should be nil. Even so, if the water tension of the soil and the concentration of the soil solution were reduced by irrigating with a water less saline than the soil solution had become from the reduction in its volume by drying, there would be, necessarily, a prompt advantage in favor of the leaves in the osmotic differential. Unless the leaves were already fully turgid such an advantage would be reflected in an increased turgescence with the turgescence reflected, in turn, by a lengthening of the leaves. This is what Gauch and Wadleigh found to be the case. Cotton, like other plants, when transferred from dilute to rather concentrated salt or sugar solutions may wilt severely and sometimes irreversibly so. But, illustrating the involvement of osmotic differentials rather than of substrate concentrations alone, is the fact that a plant may contine to grow without wilting if the same final substrate concentration is approached gradually by means of small daily increments which provide a time opportunity for salt accumulation in the leaves. The increases in the potential diffusion pressure deficits arising from extra salt accumulations in plant tissues are, of course, additive to these resulting from normal accumulations of salt and organic compounds and from transpiration. A pressure deficit in foliar tissues is transmitted to the roots as a tension in the water of the xylem vessels where it becomes a differential, at the root surfaces, with respect to the opposed water retention forces represented by osmotic and capillary forces in the soil. Others have noted that growth becomes inappreciable at moisture-supply levels higher than those at which transpiration becomes inappreciable.

In another study (36), it was found that the rate of exudation from the cut stumps of cotton plants grown on serial concentrations of chloride in sand cultures was proportional to the osmotic differentials between the exudates and the substrate solutions.

Mineral Nutrition

Many chemical analyses have been made of cotton plants grown under various conditions and stages of development. As one example, Olson & Bledsoe (37) collected, on successive dates, samples of Upland cotton growing on several soil types in Georgia. The best growth was on Cecil sandy loam. At 135 days the entire tops of plants on this soil contained, on the dry weight basis and as recalculated to m.eq./100 gm.: Ca, 57; Mg, 33; K, 25; N, 93; P, 26; and S, 20 (the S data are supplied and total P and S are ex-

pressed as PO_4 and SO_4). Hoagland's solution (×5) contains: Ca, 50; Mg. 20; K, 30; N, 75; P, 15; and S, 20. Recalling that Hoagland developed his solution on the basis of the analyses of plants, and recognizing the variations between analyses, it would seem to be indicated that cotton may be without important or peculiar distinguishing features in its general mineral relations; but it does belong among crop plants which accumulate substantial concentrations of calcium.

During the first 90 days the foregoing plants gained in mineral elements more rapidly than in dry weight, but during the first 15 days of the grand-growth period (90 to 105 days) there was a similarity between growth and accumulation. In this period the plants gained 38 per cent of their final dry weight, 36 per cent of their Ca, 53 per cent of their Mg, 38 per cent of their K, 41 per cent of their N, and 38 per cent of their P.

Of specific interest, in terms of the movement of minerals from vegetative to reproductive tissues, was the finding that the squares and bolls at 135 days (37) constituted 65 per cent of the total dry weight and contained mineral elements as percentages of the plant totals: Ca, 24; Mg, 49; K, 44; N, 55; and P, 76. Between 105 to 135 days of age there were net losses from the stems plus leaves of 13 per cent of the weight of the previously accumulated nitrogen and 30 per cent of the weight of phosphorus; during the same period there were net gains in stems and leaves in Ca by 53 per cent, Mg by 2 per cent and K by 34 per cent. Under other conditions it appears that the loss of N from the stems and leaves to buds and bolls may be somewhat greater than as stated above. As the mean of two fertilizer and two spacing treatments at Clemson, Armstrong & Albert (38) found, between August 5 and 26, decreases in nitrogen in the leaves from 3.8 per cent to 2.9 per cent and in the stalks from 1.1 to 0.7 per cent. There seems to be no agreement in favor of any particular transport form of nitrogen from the leaves and bark to the bolls; Maskell & Mason (39) in extensive discussions suggest that all forms of nitrogen, including labile proteins, are involved in nitrogen movement.

Finding that mainstalk node numbers of cotton grown in the Sudan showed the greatest increase with heavy nitrogen fertilization, and plant height the greatest increase with heavy irrigation, Crowther (40) concluded that nitrogen functions most prominently in differentiation and water in cell enlargement. The effects of the two variables on leaf weight were similar.

As a means of studying the relation of metabolites and transpiration to the uptake and transport of minerals, Phillis & Mason (41, 42) conducted a number of experiments. In the first of these they compared the accumulation of phosphorus (regarded as phloem mobile) and calcium (regarded as mobile only in the xylem) by plants having pairs of large vegetative branches but no main stem; the experiment was continued for two weeks after having bark-ringed one branch of half their plants. The ringing had only minor effects on the weight of entire plants and on the total uptake of phosphorus and calcium. But there were reductions from ringing in the order of 20 per cent in the weight of roots and in the weight of phosphorus and calcium in

the roots. Using bromine as a test element in a second experiment, it was found within 2 hr. after ringing and placing in the open that the bromine (supplied the previous afternoon) was unchanged in the tops but was 13 per cent higher in the control roots than in the roots of the ringed plants. In a third experiment, they show that with a tenfold increase in transpiration rate as a result of full rather than partial exposure to light for 9 hr., that there was 38 per cent more bromine in the totally exposed plants than in the shaded plants. Studying the relation of sugar accumulations to mineral uptake in roots, Eaton & Joham (43) found, within three weeks after defruiting, that the concentrations of sugars had tripled in the fibrous roots (relative to fruited plants) and the weight of bromine in entire plants had doubled; the effect upon nitrogen in the fibrous roots was positive but minor, and upon potassium, positive and significant.

Greater accumulations of sugars and starch in both leaves and root bark, and of total nitrogen in the leaves were found by Eaton & Rigler (6) under high than under low light intensities in both fruited and debudded plants grown at four nitrate-supply levels. The light intensity had a greater positive influence on nitrogen and carbohydrate accumulations than did debudding. Wadleigh (44), working at an intermediate light intensity, found successive increases in all forms of nitrogen in all tissues examined as the concentration of nitrate in the substrate was increased; at the same time the concentrations of starch and dextrins decreased.

In an investigation of the effects of pollination on the gain in weight and accumulation of nitrogen, phosphorus, and calcium in the ovules and carpels of young bolls, Mason & Maskell (45) found little difference during the first three days after anthesis but following this there were reductions in rate of accumulation of the minerals in the two fractions of the unfertilized bolls that were roughly proportional to the reductions in their growths. Although the differences due to fertilization were greater in the ovules than in the carpels, the effects were not large enough to suggest that syngamy altered mineral accumulation differently from growth. In observing gains and losses of nitrogen and other elements in corollas before and after the opening of the flowers, Phillis & Mason (46) found the concentrations of all but calcium to increase during the preceding night and to decrease following anthesis; the losses in total nitrogen and phosphorus were in the order of 50 per cent. Both the concentration and rate of accumulation of phosphorus was found by Biddulph & Brown (47) to decrease in floral buds and young bolls with their development; a concentration of about 1 per cent on the dry weight basis 20 days prior to anthesis, dropped to 0.6 per cent 20 days after anthesis. The authors in this report (but not in later papers) believed that phosphate transport was effected via the xylem; this view would seem to be incompatible with the fact that there is a large net export of phosphorus from vegetative tissues to bolls as the cotton crop is developed.

Mason & Maskell (45) were unable to find evidence that calcium is mobile in the phloem and literature is cited indicating a somewhat similar situation

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in other plants and a general need for a continuous calcium supply to plant roots. In one of their tables (45) the weights of elements per 100 gm. dry weight in cotton seed are indicated as percentages of the corresponding values for leaves: Si, 1; Ca, 6; Fe, 7; S, 11; Mg, 32; Na, 42; K, 103; N, 157; P, 292. Young cotton leaves were found by Phillis & Mason (48) to have less than half as much Ca as old leaves. With total Ca varying between 2 and 7 per cent on dry weight, there was always about 1 per cent that was insoluble in hot water.

Brown & Pope (49), Warner (50) and others agree that increasing percentages of phosphate in fertilizers have rather striking effects in increasing the number of early blooms and the proportion of the crop harvested at the first picking. But, as an exception, these effects do not appear in the data of Nelson & Ware (51). Going further, Crowther (52) has demonstrated a marked increase from phosphate fertilization on the boll size of Egyptian cotton. Turner (53) was unable to find a relation between bloom production and level of potash supply, but boll size was increased with the increased density of the foliage produced by high potash fertilization.

Rather abundant evidence has accumulated to show that sodium may reduce the amount of potassium required for optimal yields of cotton; in other words, a partial substitution of sodium for potassium is possible. The data in this field have recently been reviewed (54, 55, 56). At 20 locations in the Mississippi Delta where yields demonstrated no response to potassium fertilization, Landcaster, Andrews & Jones (55) obtained identical average yields of 877 lb. of seed cotton both with and without sodium additions; the exchangeable sodium in these soils varied from 40 to 170 lb. per acre. Also, on Cecil sandy loam at Clemson, South Carolina, Paden (57) found little or no increase in yield when sodium nitrate was substituted for calcium nitrate. As reported by Cooper & Garman (58), however, this substitution resulted in consistently large yield gains on heavily-leached, mineral-deficient Norfolk sand; with 0, 15, 45, and 60 lb. additions of K_2O per acre, the substitution of sodium for calcium gave increases in seed cotton per acre of, respectively, 215, 201, 187, and 182 pounds. Without potash the yield was 306 pounds and with the 60 lb. addition of potash, it was 1201 pounds. Although the authors conclude that with ample K the yield gain due to Na might have disappeared, the uniformity of the increased yields remains quite remarkable. In this connection, they note that the accumulation of Na in the plants (in the order of 10 m.eq./100 gm. dry weight) caused a similar reduction in Ca accumulation. At the 60 lb. potash level the ratio of Ca to K plus Na was 2.01 (m.eq./100 gm.) without sodium and 1.24 with sodium; they viewed the high Ca as adverse and suggest that nearly equal accumulations of Ca and K may be conducive to maximum yields. Wadleigh (59), dealing with extremely dilute nutrient solutions containing various proportions of K, Ca, Mg, and Fe in sand cultures, obtained growths of young cotton plants which appear to be in keeping with the foregoing conclusion.

At Sandhills (58), little effect of K additions on Ca accumulation in entire

tops was found but Phillis & Mason (48) found sharp and consistent reductions in Ca and Mg in the mainstalk leaves of Sea Island cotton as the potassium supply was increased from 12.5 to 200 p.p.m. In an evaluation of petiole constituents as an index to nutrient supplies, Joham (60) also obtained negative correlations between petiole accumulation of Ca and Mg and the accumulation of K. There were negative correlations, as well, between petiole P and substrate N. The petiole levels of Ca, Mg, K, NO_3, and P reflected well the availability of these ions in the substrate.

Ergle (61) has compared the effects of sulfur and nitrogen deficiencies in cotton noting the similarity in the appearance of the chlorosis caused by the two deficiencies. Soluble nitrogen accumulated excessively in the instance of S deficiency but there was no extra accumulation of sulfur compounds when N was deficient. As evidence that sulfur of proteolytic origin is not reused as is the case with proteolytic N, it is noted that under S deficiency the lower leaves remained green while the upper turned yellow, whereas under N deficiency the upper leaves gained chlorophyll at the expense of N from the lower leaves. In a later paper (62) it is shown that only 70 per cent of the sulfate sulfur that had accumulated in old leaves was translocated to new growth when the plants were transferred to S-deficient solutions; during the S-deficient period there was no decrease in organic sulfur in the old leaves and evidently none was moved out through the petioles to support new growth. It is concluded that during growth cotton requires an almost continuous external sulfur supply. Although it was shown (61) that a great part of the sulfur of deficient cotton leaves is concentrated in the chloroplasts, there is no clue to the role of sulfur in chlorophyll formation. During photosynthesis, however, Calvin (63) regards the splitting of the disulfide linkage as the means by which light energy is converted to chemical energy; others, as reviewed by Arnon (64), have presented evidence favoring the phosphate bond energy of nucleotides as being primarily involved in the reaction. In the initial paper of the series on sulfur, Ergle & Eaton (65) present data on the distribution of the various forms of sulfur throughout the cotton plant. Titratable amounts of hexose or sucrose sugar were usually not found in either the leaves or stems of S-deficient cotton plants (other plants compared) and yet the starch concentrations were only moderately depressed. The absence of sugar was regarded as indicating the possibility of carbon transport in the form of amino acids or other soluble organic nitrogen compounds. From small S-deficient plants through large plants amply supplied with sulfate, the number of bolls per 100 gm. of fresh plant weight remained constant.

It has been known for a good many years (66) that cotton on boron-deficient substrates matures few bolls. The reason for this failure is indicated in the findings of Gauch & Dugger (67) who have shown in the instance of tomato and bean plants that the translocation of sucrose, or its hydrolytic products, is dependent upon the presence of boron. Boron-deficient cotton

plants (66) are characterized by notably short fruiting branches, some buckling of the mesophyll and by the failure of young bolls to develop. Basing relative fruitfulness on the total green weight and number of green bolls on four plants, the 1930 data (66) give values of 2.3, 5.3, 6.7, 7.0, 7.6, and 5.1 for sand cultures with zero, 1, 5, 10, 15, and 25 p.p.m. of boron, respectively. The four plants in the zero boron culture had a fresh weight of 204 gm. and carried only 3 green bolls whereas those in the 10 p.p.m. culture weighed 780 gm. and had 34 green bolls (with an additional 3 that had matured). Now that it is possible to assign a role in carbohydrate translocation to boron it becomes more readily understandable why it should have such a notable effect on relative fruitfulness in contrast with the effects of drought and the levels of sulfur, molybdenum, and nitrogen.

Through a series of papers Phillis & Mason (68) offered strong evidence that carbohydrate transport in the cotton plant is in the form of sucrose. On reaching the bolls, the sucrose is rapidly transformed to hexoses; little sucrose is found in developing cotton bolls (a single exception in the literature). The possibility that sucrose is translocated as a boron ester has been discounted [(70) p. 14] because with high concentrations in the leaves of various plants only low concentrations have been found in the seeds and fruits (69). Regarding the probability that boron is moved in the transpiration stream there is an indicated resemblance to calcium. Gauch & Duggar's view that the presence of boron in cellular membranes is required for their permeability to sucrose would appear to provide a reasonable interpretation of much of the boron-deficiency literature.

Molybdenum, as studied by Joham (71) in sand cultures, has been found to accumulate more extensively in the leaves than in the stems, roots, or fruiting bodies of the cotton plant. Concentrations of 25 and 35 p.p.m. in the substrate solution were toxic. As much growth resulted in zero cultures that gave 3 p.p.m. in the leaves as in 5 p.p.m. cultures that gave 316 p.p.m. The foregoing results leave unanswered the question of whether the molybdenum requirement of cotton is very low or whether some intermediate concentration would have been more nearly optimal. Calculations of the ratios of boll weights to weights of leaves and stems provide evidence that relative fruitfulness was not altered by the molybdenum level.

In zinc-deficient cultures Brown & Wilson (72) noted that few floral buds remained alive and the few that flowered were shed as young bolls. It would thus seem that some minimum level of zinc is essential to normal fruiting activities. A basis for the foregoing may lie in Skoog's finding (73) on the necessity of zinc for the synthesis of auxin. Zinc deficiency was otherwise expressed as reduction in plant height and leaf size, chlorosis, cupping, and necrotic patches in the leaves. In a river bottom soil in Texas, Gaines (74) obtained yield increases of 19.7 and 18.8 per cent when zinc and copper, respectively, were added to a lead arsenate insecticide.

The organic acids of cotton leaves have been shown by Ergle & Eaton

(75) to be maintained substantially in excess of the concentrations required to balance inequalities in the sums of inorganic bases and acids. This was the case even though the plants were grown, after an initial period in Hoagland's solution, on such diverse single salts as ammonium sulphate and chloride versus calcium and sodium nitrate. Malic acid in this and other experiments constituted approximately 40 per cent of the total organic acid but its concentration increased (reversibly) at the expense of citric acid under drought condition. The respiration and translocation of organic acids each proceeded only 11 per cent as fast as sugar plus starch when attached and detached leaves were placed in the dark for five days.

Indeterminate Growth and Growth Correlations

Strictly speaking, the growth of the cotton plant is indeterminate, but within this conception there are variations in growth behavior which have led to the classification of some varieties as determinate and others as indeterminate. Some varieties fruit heavily during the early season after which the terminal buds become dormant and the growth of fruiting branches and rate of flower production decline; all or most of the late flowers are shed. With the opening of the first bolls, the determinate plant will usually renew its growth and flowering and a new crop will be set. The indeterminate strains, on the other hand, continue to flower throughout the summer and a good many bolls are set but at no time does the plant carry enough bolls to stop growth. The determinate cottons are often classed as early and the indeterminate as late. Between the extremes there are variations in degree. Early squares (floral buds) and short square and boll periods (76) may contribute to earliness; however, the fact remains that a determinate cotton is necessarily an early cotton, even though with the setting of a second crop there also may be many late bolls.

Long ago Ewing (77) found that when all flowers were removed from plants, starting in late July, the plants continued to flower heavily, whereas, when only half the flowers were removed flowering nearly ceased by September; the flowering rate of these latter plants coincided with that of untreated plants. Buie (78) increased the number of blooms from 14 to 21 per plant by removing all flowers as they appeared. The continuous removal of all flowers [(25) loc. cit. p. 881] from alternate fruiting branches (though it increased flowering) had no effect on the final height and little effect on yield or number of bolls. When all previously set bolls were removed by Eaton (79) from plants on July 22, growth and flowering were both stimulated and within ten days the plants were carrying more bolls than untreated plants. On August 26, seven days before again being defruited, the previously defruited plants had set (and evidently would have retained) over 50 per cent more bolls than the control plants. In the same paper it was shown in Arizona that removing all flowers from irrigated plants during the first 25 days of the flowering period resulted in larger plants, and a 20 per cent increase in the num-

ber of bolls matured. Without the advantage of irrigation, Hamner (80) obtained no loss in yield when all squares were removed for the first six weeks of the flowering period; similarly, Dunnam *et al.* (81) found no significant difference in yield when squares were removed for the first 4 weeks of flowering but there was a yield loss when they were removed for a period of 9 weeks. Gaines *et al.* (82), at two Texas stations, showed slight gains in yield when plants were not dusted during the early growth to control thrips, but were dusted later to control weevils and bollworms. Unpublished data (California) have confirmed the advantage of removing early flowers under irrigation; but when the late-season boll development is dependent upon August rainfall, the advantage of saving the early buds and flowers may often be quite real.

There is some justification for believing that inherent differences in tendency toward bud shedding must account largely for variations in degree of determinateness, but to a lesser extent boll shedding may also be involved. Comparing three varieties of cotton, Hintz & Green (76) have provided data (their Figure 1 and Table 1) which indicate that only 37, 10, and 38 per cent of the squares formed during the respective first ten days of squaring appeared as blooms during the first ten days of flowering. Kearney & Peebles (83), in studying the bud and boll shedding (as percentages of total buds) of F_3 Pima×Acala hybrids, found a highly significant negative correlation ($r-0.64$) between the shedding before and after anthesis. They point out:

> This was to be expected—. If most of the buds were shed before anthesis, obviously few would be left to shed after anthesis; and, conversely, heavy losses after the buds have reached anthesis implies that comparatively few could have been lost before anthesis.

They show by their hybrids that bud-plus-boll shedding of F_3 progenies correlated well with the shedding of the F_2 parents but that the correlation was low for buds and bolls considered separately. In the F_2 hybrids the percentage of buds shed varied from 6 to 82 per cent. Cook (84) has noted that a genetic factor is indicated in plants that abort all their buds while neighboring plants are maturing good crops. The inheritance of the bud shedding habit has also been pointed to by Harland (85). McNamara *et al.* (86) comment in connection with an extensive study of boll shedding: "No continuous record was made of small-square shedding, but in all probability there were as many floral buds (squares) shed as there were flowers produced." Loomis (87) found only 16 per cent of Acala buds developed into mature bolls whereas 46 per cent of the buds on the smaller-bolled Pima plants developed. One reason for believing that bud shedding is primarily involved in the indeterminate-growth habit lies in the fact that boll shedding is highly obvious whereas the abortion of squares commonly takes place while the buds are only a few millimeters broad and may not be noted. Boll shedding has been extensively studied, but in two investigations of boll shedding by cotton

breeders (77, 86) which involved comparisons of diverse varieties, no mention is made of a relation between boll shedding and degree of determinateness.

Growth correlations as related to fruiting are not confined to the aerial parts of the cotton plant, but are reflected as well in root development. The writer (25) removed all floral buds from Acala plants throughout a summer and increased the number of buds produced per plant from 77 to 313 and the dry weight of tops and roots per two plants in each pot from 541 gm. (including 216 gm. reproductive) to 825 gm. Bud removal increased the weight of roots from 15 per cent (fruited) to 30 per cent (debudded) of the respective total weights but the weight of roots on the debudded plants was three times the weight of the control roots. Plants grown with only two bolls (28) have been found to wilt less than fully fruited plants as a consequence of their enlarged root systems. As noted in the above paper, J. W. Neeley has observed, in the Mississippi Delta, that indeterminate strains withstand drought better than determinate strains. Apparently with the greater root development the indeterminate strains are able to obtain moisture from the deeper rootzone.

Although degree of determinateness appears to be primarily genetic it is influenced by environmental factors. In the hot, bright, and commonly humid Imperial Valley of California, indeterminate strains are apt to be highly determinate and commonly there is no regrowth for a second crop. In an experiment repeated under shades in a winter greenhouse and outdoors in the summer (6) there was a profuse shedding of floral buds under low light. The stems and leaves weighed about the same under high and low light intensities but the low-light plants produced only 10.7 flowers and of these only 12 per cent were shed whereas under high light there were 28.2 flowers per plant but 40 per cent of these were shed. A 50 per cent higher relative fruitfulness was found under the high light intensity. The foregoing values are the averages of four levels of nitrate supply; in both of the tests the low-nitrate plants were determinate in that after setting a number of bolls the terminal buds became dormant and few subsequent bolls were set.

As so capably reviewed by Loomis (88), growth correlation may be the result of simple competition for growth materials; it may depend on the formation or action of auxins or auxin inhibitors or it may depend on a hormonal control in food competition. Clear differentiations in these effects tend to be difficult to make but it seems unlikely that the dominance of fruiting activities in cotton, as shown by so many examples, can be assigned to simple competition for available food materials. A demonstration of the existence and mode of action of a mobile growth regulating material (anti-auxin) from developing cotton bolls would go far toward explaining some of the growth behaviors of the cotton plant.

It seems to the writer that the absence of flowers on day-neutral Upland cottons at low temperatures and on short-day cottons under long days is not a matter of determinateness since, in both cases, growth is largely limited

to the development of vegetative branches. Abstract translations of Konstantinov's length-of-day studies in Russia (89) report his having found the reactions of short-day cottons to artificial short days to consist of the lowering of the position of the first fruiting branches. It was observed in 1922 (7) at San Diego, California, where the day temperature rarely exceeds 80°F., that under 13-hour days and a nightly temperature of 65°F., Durango cotton produced many vegetative branches but only a few short fruiting branches. But with the same 13-hour day and a nightly temperature of 95°F. only fruiting branches were formed. These results evidently find confirmation in Biddulph & Brown's work (47). They grew a variety of Upland cotton from seed obtained in Mississippi in a winter greenhouse with thermostat control at 70°F. They note that the temperature never rose more than a few degrees above this until March, at which time the plants were large and produced their first blooms at an age over 160 days. With suitably high temperatures flowers are expected in 55 to 60 days or less after planting.

Boll Shedding and Relative Fruitfulness

Previous reference has been made to boll shedding and fruitfulness in relation to water supply. Going further in this regard, Ewing (77) compared shedding rates with soil moisture and evaporation through two seasons in Mississippi. First raising the question as to whether there was, in fact, any relationship between these variables he went on to point to several late-summer instances in which higher shedding rates corresponded to either low moisture or high evaporation; he then commented that his data "are submitted without any very definite conclusions but mainly to point out the complexity of the problem." Balls (90) noted that shedding was heaviest prior to irrigations and became excessive when the lower rootzone became saturated with the rise of the Nile. Harland (91) concluded that shedding is heaviest in the neighborhood of heaviest rainfall. In a similar connection, Lloyd (92) concluded: "Rain, if it falls in the late forenoon and probably early afternoon, causes a high degree of shedding of bolls through its destruction of pollen." Mason (93), on the same subject, noted that during the later stages of plant development augmented shedding rates followed dark, humid days during which there was generally much rain. Dunlap (33) reported that wetting open flowers twice daily with an atomizer caused only slight increase in shedding. Dunlap concluded that excessive shedding was produced: (*a*) by continuous wilting for a "few" days but not by one or two days; (*b*) by cloudy weather (he placed plants in unlighted rooms for several days); (*c*) by temperatures around 100°F. or above; and (*d*) by flooding the soil, particularly when the pots were without drains. He listed six varieties of cotton which were "less sensitive to unfavorable variations in light conditions" than four others. Of Harland's data, Ewing said: "In fact, there is little relationship to be observed between the daily fluctuations in the shedding curves and that of the other two curves presented."

In much of the foregoing, it is notable that the search for "causes" of

shedding proved to be most fruitful during the latter part of the flowering period when shedding occurs in waves and is heavy even under well-controlled conditions in a greenhouse or under some rather uniform Western field environments with frequent irrigation. Some of the so-called causes may, in one sense, be actual causes but, on more thorough examination, many appear as surface factors that tip the scale of a more basic underlying balance. As shown by a number of the graphs, the same "causes" which were associated with shedding late in the summer were usually ineffectual early in the flowering period. In keeping with previous references, Bailey & Trought (94) have developed by daily count the marked influence which bud shedding has on flowering curves. Assuming the existence of a balance between the number of leaves and the number of developing bolls carried by the cotton plant (to be referred to again in later paragraphs), a period of heavy flowering should be expected to follow, after some weeks, a period especially favorable for bud retention. If, at the time of the heavy flowering, the plant was already well fruited, it would inevitably happen that many of these flowers would be shed as young bolls and, largely by chance, the wave of shedding might or might not correspond in time with a chosen set of environmental conditions.

Beginning, perhaps, with the paper by Mason (93) in 1922 it became popular to believe that the cotton plant retains only as many bolls as it can supply with carbohydrates, nitrogen, or other nutrients. This has come to be known as the nutritional theory of boll shedding. In support of this view, but without the benefit of chemical analyses, Mason found that heavy shedding followed defoliation. In similar vein, Eaton (79) found that with the removal of all bolls from cotton plants there was a resumption of growth, more rapid flowering, boll retention, and a new crop of bolls was set. But predating the foregoing, Cook (84) wrote in 1921: "Some writers treat shedding as a disease while others consider it a normal habit of the plant to reject superfluous buds or young fruits that could not be brought to maturity." Hawkins, Matlock & Hobart (95) followed the carbohydrate levels in cotton plants under several water-supply treatments in Arizona field plantings. Although the variations in carbohydrate levels and shedding rates were for the most part inconsistent, they concluded that the percentage of shedding was dependent on the amount of plant food available for the support of the young bolls. Bearing on this subject, Ergle (96) found rather steady increases in both monosaccharide and disaccharide sugars in the entire tops of cotton plants with the advance of the summer in Texas; sugars remained rather high in the roots throughout July and August. Comparable results were obtained during a succeeding year (97) and also with five varieties (28) at College Station in 1945. In the latter instance the plants were irrigated and the carbohydrate accumulations could not have been due to a shortage of water.

From carbohydrate and nitrogen analyses of plants grown on four serial concentrations of nitrate under intermediate light intensity, Wadleigh (42)

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advanced conclusions in favor of the nutritional interpretation. Notwithstanding his assignment of shedding significance to the low N and high carbohydrate concentrations found with the low N supply and to the high N and low carbohydrates levels found with high N supply, he records that the shedding percentages were much alike under all his treatments. Computations from his data have shown that the number of bolls per 100 gm. of fresh stems and leaves changed little in going from his low to high nitrate supply; the values were 3.6, 3.5, 2.8, and 3.5, respectively. Also employing four nitrate levels, Eaton & Rigler (6) found relative fruitfulness values of 4.5, 3.8, 3.4, and 4.1 under shades in a winter greenhouse and 6.8, 6.4, 6.4, and 7.6 outdoors in the summer. Both the low and high nitrate levels depressed growth and increased relative fruitfulness in both tests. The high nitrate concentration was 64 m.eq./l. Weight of bolls was less suitable than the number of developing bolls in measuring relative fruitfulness in these tests because of the more determinate growth and higher average weights of the developing bolls in plants on low nitrate at the time the plants were harvested, i.e., only the earliest bolls were retained. In those experiments (6) the average carbohydrate levels were 2.7 times higher outdoors under high light than they were under the winter shades. Although still assuming that carbohydrate levels should influence the number of bolls retained, the authors in summarizing their data raised the question as to why the carbohydrates under high light were not reduced to the low-light levels before shedding occurred. The nitrogen levels in the roots were about the same in the winter and summer plants. In a subsequent experiment with irrigated cotton in field (99) light was reduced by shades to 32 per cent. In this experiment, as in the one just mentioned, there was little effect of reduced light on the fresh weight of the stems and leaves. The loss in total weight was due to the reduction in boll weight. The total above-ground growth under the shades was 60 per cent of that in the open. By extrapolation it was deduced that 54 per cent of full exposure would have been required for a total plant weight equal to that in the open. This value is in keeping with a value of 52 per cent obtained by Thomas & Hill (100) for irrigated alfalfa. In regions of rather low temperatures it seems possible that what has been regarded as causal relations between shedding and cloudy weather are sometimes actually due to the associated low temperatures.

Eaton & Ergle (98) measured the carbohydrate and nitrogen levels in leaves, stems and 13-day-old bolls of early medium, and late plantings of Acala and Stoneville cotton at Shafter, California; the experiment was repeated with a high, medium, and low water supply. When last sampled in mid-August there were no appreciable differences in the carbohydrate and nitrogen concentrations between the first plantings which were shedding most of their bolls and the late plantings which were retaining their bolls. In other experiments (98), girdling and daily spraying with sucrose and urea in various treatment combinations always increased shedding, notwithstanding some marked increases in nitrogen from the urea spray and in carbohydrate

accumulation from girdling. There was no evidence that any of the sucrose sprayed on the leaves was accumulated. In the light of these results, it was concluded that the explanation of boll shedding should be sought in other directions.

The uniformity of relative fruitfulness data with varying growths under the influence of varied moisture, nitrogen, sulfur, and molybdenum supplies together with the fact that boll shedding is nominal until a good many bolls have been set provided the basis for the suggestion (98) that a causative balance might exist between the production of auxin or a precurser in cotton leaves which was balanced against a mobile antiauxin or inhibitor produced in developing cotton bolls. The requirement for mobility originated from the observed scattering of bolls on the cotton plant and from the fact that about the same number were produced when the setting occurred at random positions and when limited to alternate fruiting branches. In a later paper (99), some of the published as well as unpublished results from hormone studies regarded as bearing on the foregoing theory were reviewed. Among these, H. R., Carns had reported in a defoliation conference that small cotton bolls showed no auxin transmission to agar blocks but when similar bolls were placed on blocks containing 100 μg. of IAA,[2] the IAA activity disappeared. Heavy boll shedding is commonly coincidental with growth inhibition. This would indicate that the hormone stimulus responsible for shedding is an antiauxin, or is produced simultaneously with an antiauxin. In one experiment (see 104), weekly spraying with 20 p.p.m. of 4-chlorophenoxyacetate resulted in leaf symptoms, increased vegetative growth, and sharply reduced relative fruitfulness. In later studies, on the other hand, no effect on relative fruitfulness was found to result from spraying three times weekly with 2500 p.p.m. of *trans*-cinnamic acid, either in the open or under muslin shades.

Although the movement of an antiauxin from massive old bolls might be more difficult to demonstrate than such movement from young bolls, the growth relations of the cotton plants indicate that all bolls may be involved and possibly to a similar extent. In determinate cottons both renewed growth and boll setting occur coincidentally with the opening of the oldest bolls even though no young bolls may have been set for a number of weeks.

It has been noted (99) that without the protective mechanism of boll shedding, the cotton plant would undoubtedly overfruit and, in accord with the behavior of many horticultural plants, produce smaller fruit with the probability of poor fibers. With weak or short fibers there would be serious adverse effects on the spinning properties of cotton.

Results from two additional studies bear on the foregoing discussions. Kearney (101) working with Pima Egyptian cotton in 1921 and 1928 removed, on the day of anthesis, the 3-bracted involucre which subtends the flower in all species of *Gossypium*. During the two years, the percentages of

[2] IAA, Indole-3-acetic acid.

unmutilated (control) bolls retained were, respectively, 3.3 and 7.0 per cent greater than those that were mutilated; the latter difference was significant. Cutting away longitudinally one half of each new leaf at weekly intervals starting at the time of the first flowers (99) caused reductions in plant height by 28 per cent, yield by 14 per cent, and sugar plus starch in the main stems by 42 per cent. There was little difference in sugar plus starch in the leaves or young bolls as a result of the leaf clipping. The leaf blades constituted about 40 per cent of the weight of leaves plus stems both with and without leaf clipping. The plants were smaller and the leaf clipping favored the entrance of light into the plants, i.e., there was less self-shading. Relative fruitfulness was increased by the leaf clipping from 4.6 to 5.1 (LSD 1.0). If, as has been thought, the carbohydrate level in the middle third of the main stalk provides a sufficient index to the supply for transit to the bolls, then under the nutritional interpretation, the leaf clipping should have resulted in a reduced relative fruitfulness but this was not the case.

Applications of various growth regulators (102, 103, 104, 105, 106, 107, 108, 109) have influenced the growth of cotton plants and (when the concentrations were high enough) they have resulted in malformations of the leaves and bolls. As shown by McIlrath & Ergle (109) with 2,4-dichlorophenoxyacetic acid (as well as with such compounds as maleic hydrazide, *trans*-cinnamic acid, *n*-naphthyl maleimide and triiodobenzoic acid in unpublished data) there is a marked stimulation of vegetative-branch formation with reduced growth when seedlings or young plants are treated, but after a good many fruiting branches have started to develop the increases were minor. Also, as shown in the next section, IAA (indoleacetic acid) may retard leaf abscission. But, independent of major effects upon growth, such materials have been without pronounced effect on fruiting behavior. The fact that in reasonable concentrations these materials tend to have little influence on boll shedding or on the position of the lowest fruiting branches, provides additional support for the view (104) that the fruiting branches of the cotton plant are, in fact, homologous to the inflorescences of other plants.

In the light of the available evidence it seems necessary to conclude that the cause, or causes, of boll shedding are unknown. This is the case even though boron and zinc deficiencies cause abortions. One of the salient features of the fruiting of the cotton plant lies in the fact that within a variety and climatic complex (and largely independent of the nutritional status) the number of bolls that are set tend to be proportional to the weight of leaves and stems, provided that the spacings of the plants are the same and day-length and temperatures are suitable for the formation of fruiting branches. The stimulatory mechanism responsible for the formation of fruiting branches, or inflorescences, as is the case with other plants, remains as a general problem. A more specific problem in the instance of cotton is the nature of the stimulus or balance that prevents over-fruiting through the agency of boll shedding.

The boll and fiber properties of cotton plants are remarkably stable.

Although drought may result in substantial reductions in boll size and in fiber length and weight-per-inch, it has seemed notable that limiting the number of bolls carried by cotton plants to two, with consequent gains of 20 to 45 per cent in the carbohydrate concentrations in the stems, resulted in only minor gains in boll size and fiber length (28). Likewise, reductions in stem carbohydrates by 42 per cent by cutting away one half of each leaf (with no change in relative fruitfulness) and by 37 per cent under shades (with a reduction in relative fruitfulness) had only small effects on the foregoing properties (99). Carbohydrate levels as increased by treatments, as well as higher rates of carbohydrate utilization among five varieties, however, were found to improve fiber strength (28, 99). Literature dealing with some phases of the growth and composition of the lint cell has recently been reviewed by O'Kelley & Carr (110).

Defoliation

With the advent of mechanical harvesting of cotton, extensive new defoliation research and new industries came into being. Apparently the first defoliation of field cotton was by Hall & Harrell (111) in 1938. Using calcium cyanamide dust they dropped the leaves of rank cotton to promote aeration and thereby the suppression of boll rots. Gull (112) next reported on the use of cyanamide as a leaf-abscission agent to improve the action of picking machines and to avoid stains and leaf trash in the seed cotton.

The diverse chemical nature of some of the commercial and experimental defoliants affords few clues to the physiology of defoliation: diodium-3, 6-endoxohexahydrophthalate (Endothal); magnesium chlorate hexahydrate (De-Fol-ate); sodium dichromate (Penn salt 788); 1,4-butyne diol (Penn Salt NP-1098); Sodium chlorate-pentaborate (Shed-A-Leaf); monosodium cyanamid (American Cyanamid X-5); sodium chlorate-pentaborate tetraborates (Pacific Coast Borax Cotton Defoliant); and sodium ethyl xanthate (S.E.X.). Within this series there is little evidence of a correlation between chemical structure and the physiological activity of the various compounds but many tend to be rather strong oxidizing agents. Warburg respirometer tests by Hall & Lane (113) showed that respiration was increased and in some cases doubled by the application of Shed-A-Leaf and some of the other defoliants.

As a part of the background for the current physiological interest in the action of defoliants on cotton, it has been shown by a series of investigations with other plants that ethylene gas and the ethylene-containing emanations from plant tissues produced petiole abscission. It has been shown further that natural leaf abscission is retarded or inhibited by applications of IAA. In the same connection a measure of significance has been attached to the decline in auxin or its precursor in leaves with age and approaching senescence. Theoretical considerations led Gawadi & Avery (114) and Hall (115) to suggest that leaf abscission is controlled by a hormone-ethylene or auxin-ethylene balance. A lanolin paste of naphthoxyacetic acid applied to the cut

surface of debladed cotton petioles (114) did not retard abscission as it did in poinsettia but it did inhibit the formation of the abscission layer. Using biological tests Jackson (116) developed good evidence that an emanation regarded as ethylene was greater from cotton leaves treated with any one of seven defoliants than from untreated leaves. The gas from the treated leaves when transferred to bell jars caused abscission. When the gas was transferred to enclosed petioles, adjacent unenclosed petioles also abscised. At about the same time Hall (115) found by chemical methods that treating leaves with each of two defoliants increased measurably the production of ethylene. Both of these defoliants inhibited respiration temporarily and then increased the rate relative to control leaves. Hall treated debladed cotton petioles of preflowering plants with various ratios of IAA to Endothal, ethylene chlorhydrin, and *trans*-cinnamic acid in lanolin and found in some ratios that a substantial measure of protection was afforded; IAA alone prevented abscission. Little change in abscission was observed by Lane (117) when the ends of debladed petioles were soaked in concentrated solutions of maleic hydrazide, *trans*-cinnamic acid, tri-iodobenzoic acid and nicotine sulfate.

With variations in degree, defoliants have been found to be more effective on cotton plants growing in moist than in dry soils, under some circumstances in rich than in poor soils, and on well-fruited than on poorly fruited plants (118). Each of these conditions tends to reduce the carbohydrate levels in cotton plants. In a greenhouse experiment using the foregoing treatment variables Hall (119) found an inverse correlation between starch levels and the effectiveness of cyanamide defoliants. By statistical analyses nitrogen in the leaves appeared not to influence defoliability. Subsequent investigations in the field, not all of which have been published, have cast doubt on whether starch levels are directly related to the reaction. Inconsistent results were obtained by Hall & Lane (113) both in tests with potted plants and at three Texas field stations growing different varieties of cotton under rather different climatic conditions. In all of these tests there was a consistent loss of leaf-blade starch following the application of the defoliants. They concluded that the changes in carbohydrate fractions during the defoliation process were more important in determining abscission than the original carbohydrate levels. Lane (117) found that abscission proceeded twice as fast at 30°C. as at 20°C.

Differing from results with other species and with higher sucrose concentrations in sprays, Lane & Hall (120) improved defoliation when 2.5 per cent of several sugars were added to solutions of (*a*) sodium chlorate-pentaborate, and (*b*) disodium 3,6-endohexahydrophthalate plus ammonium sulfate, into which the cotton leaves were dipped. The results are discussed in terms of improved translocation and the sugars may have increased the retention of the defoliants, but in this connection it is to be noted that the two defoliants (one containing and one without boron) were about equally effective under the various conditions and pretreatments of the plants. A leaf dipped in a 2.5 per cent sugar solution gains about 20 per cent in total

fresh weight. Even if the sugar was all absorbed, only about 20 per cent would be added to the amount of sugar commonly present in cotton leaves during the afternoon hours.

Working with a range of cotton species and varieties, Brown & Rhyne (121) noted marked inherent differences in defoliability. They also concluded that the stage of boll maturity has an important influence on defoliation. In one of the experiments boll ages were adjusted into groups by prior defruitings. In their data, as has been the case in experiments of others where heavily fruited plants were compared with defruited or insect-damaged cotton, the abscission percentages are based on the total number of leaves rather than on those of like ages. It is well known that defruiting is followed by new growth and that expanding leaves defoliate poorly. This criticism is not intended to discount the possible role of bolls in the hormone relations of defoliation but it does bring to mind rather striking evidence by Fisher & Loomis (122) on the need of differentiating between the effects of young and old leaves (auxin and antiauxins) in the flowering of soybeans.

It remains largely unknown whether boll shedding and leaf abscission are initiated on the same physiological basis but there are a number of important differences in the age relations. With or without defoliant treatment bolls more than ten days old or leaves young enough to be expanding rapdily are usually retained on plants unless the defoliant is strong enough to cause burning. A heavy shedding of young bolls normally occurs in August well in advance of the abscission of any but the oldest leaves. Floral buds rarely abscise during the ten days preceding anthesis.

Seed Composition as Influneced by Fertilizers and Environment

There is quite an extensive literature on the composition of cotton seed and on nutritional and other factors that influence seed weight and the percentages of oil and nitrogen. Portions of this literature were reviewed and new data presented by Tharp *et al.* (123) in 1949. Guthrie *et al.* (124), citing 119 papers in 1949, provided tabular data on the occurrence and concentrations of both organic and inorganic constituents found in the entire cotton seed and in the various parts including the extracted meal or cake. Data for cotton fibers are reported, also, with 85 citations.

In the cotton seed kernels (which constitute about 55 per cent of the weight of fuzzy seed) it is usual to find (123) in the neighborhood of 35 per cent of oil and a similar amount of protein ($NH_3 \times 5.13$) with the result that any large percentage increase in either of these constituents is apt to be accompanied by a reduction in the other. Seed in late bolls are often higher in oil, and sometimes in nitrogen as well, than early bolls. In highly infertile soil and under drought conditions, the seed are less well filled and contain more fibrous material in the kernel with the result that reductions occur in oil and protein. Plants supplied with insufficient water over a period of three years (27) were found to contain 6, 2, and 20 per cent less oil in their seed

than was found in seed from irrigated plants. In the same experiments seed from plants limited to only two bolls contained, in two tests, 9 and 6 per cent less oil and 5 and 1 per cent more NH_3 than seed from well fruited plants. Both the drought and partial defruiting increased the carbohydrate and nitrogen levels in supporting the plants.

It has been found in many instances (123) that nitrogen fertilization increases the weight of the kernels and their percentage of nitrogen whereas the oil content tends to be reduced slightly. Phosphorus fertilization has usually been without effect on oil or nitrogen composition. Potassium applications usually result in a marked increase in oil content accompanied by a slight reduction in nitrogen. Sodium applications have induced some effects in the direction of those produced by potassium. Calcium and magnesium applications have had little effect upon composition.

Effects of Spacing on Growth and Fruiting and on Boll and Fiber Properties

The subject to be reviewed in this section has been reserved until the last because so many of the reactions of cotton plants to close spacing bear on matters previously discussed. Rather than to attempt a coverage of the extensive literature, it seems preferable to summarize briefly a few of the more salient responses to close spacing on the basis of the review contained in a comprehensive article with 45 citations by Peebles, Den Hartog & Pressley (125). Examples of the magnitudes of the various responses to close spacing under irrigation are then drawn from the experiments by Peebles *et al.* in Arizona (125) and under drought conditions from a single experiment in Texas by Hervey, Fisher & Johnston (126).

The spacing of cotton plants a few inches apart in rows versus plants spaced a foot or more apart results in a marked suppression of the growth of vegetative branches; shortening and reduction in the number of nodes of fruiting branches; the appearance of the first boll on a higher than usual fruiting branch and, commonly in taller plants, greater weights of plants per acre; increased numbers of bolls per acre; and often (but not always) in reductions in boll sizes. A decrease in the number of locks per boll has also been noted. Under the diverse conditions represented in the many experiments reported in the literature, there has been a lack of uniformity in conclusions as to optimum spacings for highest yields and in the effects of close spacing on earliness. As discussed by Peebles *et al.* (125), close spacing exerts two opposite effects on crop maturity. When plants are closely spaced, few fruiting branches develop beyond their first node which contributes to earliness since the interval of flower appearance is much shorter between first nodes of successive fruiting branches than for the successive nodes on a particular fruiting branch. The first boll, on the other hand, appears later and higher on close spaced plants than on wide spaced plants which results in a delay of maturity.

Comparing spacing distances from 2 to 6 inches with those of 12 to 16 inches, Peebles *et al.* (125) found, under irrigation, an average increase of 9.5 per cent in the yield of cotton lint in seven Upland cotton experiments (each of which included a number of varieties), and in a like number of experiments with American-Egyptian varieties the increase was by 12.9 per cent. Neither the weight of lint per seed (lint index) nor the weight per seed (seed index) was influenced appreciably by spacing. There were average reductions in the weight of seed cotton per boll of 4.1 per cent in the Upland cottons and of 3.8 per cent in the American-Egyptian cottons. Also, there were reductions in the number of seeds per boll in the Upland cottons of 3.9 per cent and in the American-Egyptian of 5.5 per cent. These average reductions in weight of seed cotton per boll and in the number of seeds per boll would seem to be similar enough to indicate that with ample water supply spacing influences boll size primarily as it influences the number of seeds per boll. In the dry cotton in Texas (126) there was a reduction in seed cotton per boll of 26 per cent with close spacing. This reduction, it would appear, was due primarily to a more limited water supply in the close spaced than in the wide spaced cotton; the dry weight of burs, leaves, and stems per acre in the close spaced cotton was double that of the wide spaced cotton. Both seed index and lint index commonly have been found to be reduced in similar proportions under drought conditions. In Texas stem diameters of 0.69 cm. were found with 2-inch spacing and of 1.06 cm. with 11.5-inch spacing.

Close spacing has resulted in greater increases per acre in the weight of plants than in the weight of seed cotton with the result that relative fruitfulness is reduced. In the Arizona investigations (125), measurements were made after picking of per-acre field weight of stalks and burs in two experiments with Upland cottons and in three with American-Egyptian cottons. In the Upland experiments (comparing spacings of 2 in. versus 14 in.) the close spacing reduced relative fruitfulness by 9 per cent in terms of the ratios of seed cotton to stalks and burs. The corresponding average reduction in relative fruitfulness for the American-Egyptian cottons (spaced 4 or 6 in. apart versus 20 or 22 in.) was 4 per cent. Under the exceptionally dry conditions in Texas (126) during late June, July, and August, there was a seed-cotton increase (in a 2-in. spacing versus an 11.5-in. spacing) of 32 per cent and in the oven-dry weight of entire plant tops (less seed cotton) there was an increase of 101 per cent. The ratio of the weight of seed cotton to weight of the tops less seed cotton was reduced by 26 per cent by close spacing.

Earliness as judged by the percentage of the crop derived at the first picking was not influenced in the Upland cottons in Arizona by spacings between 6 and 16 in. but there were marked reductions in the proportion of of the crop derived at the first picking with 4 and 2 in. spacings. The earliness of the American-Egyptian cotton improved progressively as spacing was reduced to 6 in.

XUM

Except for some coarsening in the fibers of the American-Egyptian cottons spaced at 2 and 6 in., close spacing was without effect on fiber length and had little effect on the fineness of the fibers in Arizona. Spacings of 6 in. or less resulted in a mean reduction in the fiber strength of the Upland cottons of 2.6 per cent and in the American-Egyptian cottons of 1.3 per cent. The yarn strength of the Upland cottons (measured in one experiment) also decreased as the spacing interval was reduced from 14 in. to two in. Although a reduction 2.6 per cent in fiber strength may appear to be minor, the authors observe, "such weakening is not to be regarded lightly; from the profit standpoint to the grower, however, it would seem to be more than offset by the large gain in yield." The dense shading of the ground in the close spaced cotton was noted and the foregoing reduction in fiber strength would be in harmony with the loss of strength previously found (99) under muslin shades. Water deficiencies often have been found to increase fiber strength but with the strength increases there were reductions in fiber length.

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PATHOGENESIS IN THE WILT DISEASES[1,2]

By A. E. Dimond

The Connecticut Agricultural Experiment Station, New Haven, Conn.

The vascular wilt diseases provide an opportunity for examining two kinds of physiological problems. First, because they disturb the normal water economy of plants, these diseases can be studied with a view to examining such problems as absorption of water by damaged roots, the ascent of sap through mycelium-invaded xylem, and the effect of disease on the stomatal and osmotic mechanisms of leaves. Second, this group of diseases is caused by a range of bacterial and fungus pathogens which invade the xylem early and limit their host invasion primarily to this tissue until the host is dying. In the many hosts attacked, the symptoms are strikingly similar. The close similarity of symptoms suggests that a variety of pathogens, growing in many hosts, produces wilt diseases through a limited number of biochemical mechanisms. The latter argument is presented for simplicity's sake in terms of diseases caused by *Fusarium oxysporum* and *Verticillium*. The reader will be aware of the extent to which these arguments apply to other pathogens.

INVASION OF THE HOST AND SYMPTOM DEVELOPMENT

Infection of the host by *Fusarium* and *Verticillium* occurs through roots, the fungus penetrating fairly directly to vascular tissue. Thereafter further mycelial growth is almost always restricted to conductive elements of the xylem, passage from one element to another being through pits (1, 2). Mycelium continues to grow through root and stem and in many cases it eventually appears in petioles and the growing point. In late stages of these diseases, the fungus causes disintegration of stem tissues generally and may fruit on the surface after the plant dies. By this time, extensive rotting of roots may have occurred (3, 4).

The earliest symptom of *Fusarium* wilt of tomato appears within 48 hr. of root inoculation. By then the ultimate veinlets of leaves may have become translucent [Foster (5)]. A few hours later the oldest petioles become epinastic. Gradually epinasty appears on all leaves [Wellman (6)]. Occasionally, adventitious roots may form on the stem, keeping pace with the yellowing of the leaves [Fisher (7)]. Similar formative symptoms are known in cabbage yellows, celery yellows, bacterial wilt of potatoes, Dutch elm disease, *Verticillium* wilt of mint and, in *Fusarium*, severe wilt of peas (8 to 12).

[1] The survey of the literature pertaining to this review was completed in August, 1954.

[2] The following abbreviations will be used: PG, polygalacturonase; PME, pectin methyl esterase.

In many wilt diseases, however, these very early symptoms have not been reported.

The obvious disease symptoms appear from two to four weeks after the plant is inoculated. Vascular bundles become conspicuously disclored and the leaves wilt. The oldest leaves wilt first and successively younger ones become symptomatic in an orderly manner. Vascular discoloration, after developing through the root system to the base of the stem, similarly appears in successively younger petioles and stem tissue until the whole stem is involved. At first the leaves wilt[3] in the day and recover at night, the symptoms gradually becoming more severe as irreversible withering[3] develops. Leaves then become yellowed and desiccated[3] or are abscised. The infected plant shows all the external symptoms of a gradually increasing water shortage.

Although the incubation period varies, symptoms of many other diseases are essentially the same as for the *Fusarium* and *Verticillium* wilts, e.g., Dutch elm disease, *Cephalosporium* wilt of elms, oak wilt, and the bacterial wilts of potato, tomato, and tobacco.

Obviously, two kinds of symptoms can be distinguished: formative symptoms such as epinasty and adventitious root development, and the later-appearing symptoms of leaf wilting and the associated vascular discoloration. The origin of the formative symptoms will be discussed first.

EVIDENCE ON THE ORIGIN OF EPINASTY AND RELATED SYMPTOMS

Ethanol will cause epinasty in tomato shoots and the tomato wilt *Fusarium* produces ethanol in quiet cultures [Ludwig (13)]. However, the amount of ethanol in the tracheal fluid of infected plants is too low to cause epinasty [Dimond & Waggoner (14)].

Sufficient ethylene is produced by *Fusarium* in culture and in diseased plants to cause epinasty. Presumably, ethylene is moved with the transpiration stream because all leaves may become epinastic. Ethylene causes yellowing but not abscission of tomato leaves. It may also stimulate formation of adventitious roots. Such symptoms are ascribed to the ethylene formed in diseased plants [Dimond & Waggoner (14)].

INTERRELATIONS OF THE DOMINANT SYMPTOMS

The balance of this paper is concerned with the origin of vascular discoloration and the leaf symptoms. Possibly vascular discoloration produces a dysfunction of water-conducting elements which, in turn, leads to desiccation of leaves. A test of this is the extent to which the two symptoms are correlated with each other and with the presence of mycelium. In *Fusarium*

[3] Wilting in this paper means simple loss of leaf turgor without complete dehydration. Withering refers to crisp drying, and desiccation refers to symptoms of water shortage other than a reversible loss of turgor.

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wilt of tomatoes vascular discoloration occurs only a few millimeters in advance of mycelium, and the distribution of leaf symptoms is very highly correlated with that of vascular discoloration [Dimond *et al.* (15)]. When infection in a plant is one-sided, only the vascular bundles containing mycelium become discolored and symptoms of water shortage develop only in leaves dependent upon these bundles for their water.

Experimentally, however, vascular discoloration and wilting can be produced in infected plants at a considerable distance from mycelium. Eggplant, tobacco, and *Physalis* are all immune to *F. oxysporum f. lycopersici* and can be grafted as scions on tomato roots. Such scions show discoloration of stems and desiccation of leaves five to ten days after tomato roots are inoculated, but none if roots are not inoculated. Although mycelium is abundant in roots, it cannot be isolated from the scions [Davis (16)]. In the *Verticillium* wilts also, discoloration can occur where there is no mycelium [Nelson (12); Rudolph (17)].

Moreover, the correlation between leaf symptoms and discoloration of the vascular bundles is not perfect [Nelson (12), Clayton (18), Haymaker (19)]. Such failures in the correlation suggest that these two symptoms may arise quite independently of each other.

EVIDENCE ON THE CAUSE OF DESICCATION SYMPTOMS IN LEAVES

The diseased plant obviously has a disturbed water economy. The rate of wilting and the severity of symptoms is directly related to the vapor pressure deficit of the atmosphere, as measured by the rate of water loss from white porous clay cups [Porter (20)]. Diseased plants cannot reduce the moisture content of a soil to the same level that healthy ones can [Melhus, Erwin & Van Haltern (21)]. This can result from impaired absorption of water, dysfunction in conductive elements, impairment of the osmotic system in spongy parenchyma of the leaf or damage to the stomatal mechanism so that stomates remain open.

Absorption of Water by Diseased Roots

When plants have been infected for some time, the root system becomes heavily discolored and *Fusarium* causes considerable root damage. Orton has suggested that this impairs the ability of the roots to absorb water and so leads to wilting and desiccation of leaves (22). However, wilting usually appears long before root damage is extensive. The roots of *Verticilium* infected plants sometimes show no external symptoms and die only when the plant dies [Nelson (12); Rudolph (17)].

Injury to roots apparently does not reduce absorption of water from the soil. Thus, Kramer decapitated plants at the base of the stem, connected the root system to a vacuum pump, and measured the rate of water absorption in terms of the water collected above the cut end of the stem. Water absorption by a dead root system was faster than by a living one (23). In

an intact plant a comparable system is involved. There is a pressure deficit in the vascular bundles caused by transpiration of water from leaves.

Roots of tomato seedlings absorb water normally after they have been exposed to culture filtrates of the tomato wilt *Fusarium* [Ludwig (24)]. Extensive root damage occurs when infected plants are grown in a warm (27°C.) moist soil. However, if the air temperature is kept cool (17°C.) the fungus advances only to the base of the stem and no leaf symptoms develop [Clayton (25).] Evidently the root system continues to supply the water requirements of stems and leaves in spite of any root damage caused by disease. Root damage may contribute to but is surely not the sole cause of leaf symptoms.

Impaired Water Transport in Conductive Elements

There is considerable evidence for a reduction in rate of water movement through vessels of diseased stems. Under a constant pressure head, *Fusarium*-infected cabbage stems transport water at about 12 per cent of the rate in healthy ones [Melhus *et al.* (26)]. Diseased tomato stems move radiophosphate at from 2 to 4 per cent of the rate that healthy ones do [Dimond & Waggoner (27)]. A number of proposals have attempted to account for the reduced water flow, some more plausible than others.

The gas embolism theory proposed that CO_2, produced by the pathogen in tracheal elements, caused a gas lock in vessels [Tochinai (28)]. The theory, based solely on the observation that CO_2 is produced in cultures, was not tested by comparing the number of gas emboli occurring in healthy and diseased plants. The percentage of vessels invaded by the pathogen is always small. Also the amount of CO_2 produced per vessel might be soluble in the rapidly moving water of the transpiration stream unless other factors impede water flow. Such emboli would be small and probably would dissolve again [Dixon & Blackman (29)]. In healthy woody plants, many vessels are gas-locked and this is probably also true of herbaceous plants [Preston (30)]. If so, the amount of gas liberated by the pathogen would be small relative to that freed by living host cells.

A viscous tracheal fluid would impede flow in conductive elements and, in certain bacterial wilts, the tracheal fluid is demonstrably higher in viscosity than is that of the healthy plant. However, the tracheal fluid of *Fusarium*-infected tomato plants has the same viscosity as that of healthy plants and of water [Dimond & Waggoner (27); Waggoner & Dimond (31)].

The bundle-plugging theory proposes that wilting symptoms result from plugging of conductive elements by the pathogen itself or its products [Stewart (32); Smith (33)]. Materials which may be responsible for such plugging include mycelium, dislodged conidia, tyloses, gums, and other hydrophilic compounds. The role of mycelium in obstructing water flow in vessels has been debated. Generally, many more vessels are free of mycelium than are invaded in diseased plants and mycelium alone cannot account for vascular occlusion (11, 12, 17, 34 to 37). In celery yellows, *Fusarium* may

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cause extensive occlusion of root vessels long before the plants wilt [Nelson, Coons & Cochrane (38)]. Watermelon wilt is apparently an exception and mycelium is said by Wilson occasionally to cause sufficient plugging to induce wilting, particularly in seedlings (2).

In segments of healthy tomato stems water flow obeys Poiseuille's law. In *Fusarium*-infected stems, water flow under constant pressure head is about 5 per cent of that in healthy ones. This is much less than the estimated conductive capacity of the vessels in which mycelium is absent [Ludwig (24)].

The extent to which mycelium in a vessel interferes with water flow has been measured by means of scale models, designed to have the same Reynolds number as for water flow in the plant. Evaluation of the Reynolds number quickly shows that the transpiration stream has laminar and not turbulent flow. Therefore, obstacles in vessels such as mycelium, tyloses, perforation plates, and pits do not interfere by increasing turbulence but instead cause a small resistance to flow by the frictional drag which they create. Even if all vessels contained mycelium, the resistance to flow thus created would contribute to but could not be the sole cause of water shortage in the diseased plant [Waggoner & Dimond (31)]. Actually, the reduction in flow created in diseased stems by mycelium, considering its distribution, is probably less than 5 per cent.

Conidia are sometimes formed by mycelium in a water-conducting element. Such conidia may be released and migrate with the transpiration stream [Millikan (39); Yoshi (40)]. Such a mechanism may be involved when an entire plant wilts suddenly. In such cases the conidia do not appear to cause serious wilting by blocking the conducting element mechanically, but rather by their subsequent growth, causing new loci of infection and resulting in a systemic and simultaneous invasion of the entire plant. For example, after detached shoots absorb spores of *Verticillium* or *Fusarium* from water suspensions, they can be rooted. After the normal incubation period, the entire rooted cutting collapses and is then invaded by mycelium throughout the water conducting system [Keyworth (41); Scheffer & Walker (42)].

Tyloses in vessels could reduce water flow. Certain oaks and the cucurbits form tyloses readily whereas other plants do not. In oak wilt, plugging by tyloses and gums is extensive and thought to be a cause of wilting [Struckmeyer *et al.* (43)]. In watermelon wilt, Sleeth observed tyloses in from one-fourth to all vessels of infected but few in healthy plants (44). Yoshi questioned whether they were extensive enough to cause wilting (40). In many other wilt diseases tyloses occur rarely if at all, e.g., tomato wilt and *Verticillium* wilt of mint [Nelson (12); Ludwig (24)].

Occluding substances have been observed in vessels of *Verticillium*- and *Fusarium*-infected plants. This material was visible in fresh sections but disappeared when preparations were fixed and stained [Ludwig (24); Bewley (45)]. Such behavior may account for the failure of others to observe occluding materials in the sections they examined (12, 37, 46, 47). The gummy

occluding material is translucent and grey and appears early in diseased plants. After the onset of wilting, this material becomes an opaque brown or black and is seen in fixed and stained sections as "gum" (2, 12, 17, 24, 45).

The nature of the substances causing occlusion in early stages of wilting is unknown. Compounds suspected of being involved include the pectic substances, gums, and other polysaccharides. Sufficient polysaccharides are produced by *Fusarium* in culture to cause wilting of plant shoots which absorb the filtrate [Thomas (48); Dimond & Waggoner (49)]. Pectins may also be involved [Ludwig (24); Hursh (50)]. *Fusarium* produces pectic enzymes which may liberate large molecules of partially hydrolyzed pectin into vessels [Waggoner & Dimond (51)]. [See also Gothoskar *et al.* (52); Winstead & Walker (53).] As gum forms in vessels, the adjacent pectic middle lamella disappears where it is exposed at pits [Higgins (54)]. Galacturonic acid, araban, and galactan are associated both in pectin and certain plant gums [Bonner (55)]. Gums may be formed from products of pectin breakdown [Higgins (54); Norman (56)].

Water transport through plant stems and petioles occurs through the primary xylem in young plants and through secondary tissues if and when they develop. In the tomato plant the primary vascular skeleton is known to be comparable in all respects to that of the potato plant [Ludwig (24); Artschwager (57)]. Vascular bundles are completely interconnected in the stem so that if one bundle is severed, the tissue supplied by it continues to receive water. Ludwig demonstrated this dramatically by making two notches at the base of young tomato stems, so placed that only one of the six vascular bundles continued to supply water to the top. Even when such plants were placed under conditions favoring high transpiration, they did not wilt (24). As this experiment demonstrates, there is a considerable margin of safety in the capacity of the tomato stem to conduct water beyond the ordinary demands of absorption and transpiration. The margin of safety is increased by secondary thickening.

In petioles the margin of safety is small because leaf traces are not interconnected and in the petioles of many plants no secondary thickening occurs. A dysfunction in petiolar conductive elements is therefore more critical than one of comparable magnitude in the stem.

Plugging along the length of conductive elements of the stem can lead to localized water shortage of tissues, but the by-passes offered by the vascular skeleton keep to a minimum the amount of tissue affected. If plugging occurs in all bundles of the stem, the entire top should wilt at once. If a petiole becomes plugged, however, only the individual leaf is affected. If equal lengths of a healthy and diseased petiole are subjected simultaneously to an equal pressure gradient, dye usually fails to pass through the diseased petiole by the time it has passed through the healthy one [Scheffer & Walker (58)]. Mycelium grows up the stem from the roots and leaves wilt as each petiole is invaded, probably as a result of the biochemical processes suggested

above. In this way one can account for the characteristic progressive symptom development of the wilt diseases in which the oldest leaves are the first to show water shortage and the successively younger ones then become involved (58).

The conductive dysfunction in the stem, while severe, may not limit the water supply to leaves to the point of producing desiccation symptoms except in old infections. Such a view of conductive dysfunction in the stem can be tested experimentally by examining the stomatal behavior of nonsymptomatic leaves on diseased plants.

Effects of Disease on the Stomatal Mechanism and Transpiration

If water movement through the stem is slowly decreased as disease progresses in a plant, a water shortage in leaves should develop which becomes ever more acute. This should produce drought hardening. Nonsymptomatic leaves on diseased plants supplied with ample water should behave similarly to leaves on healthy drought-hardened plants. A comparison of the two kinds of leaves showed that in both the stomates remain closed during the day. Nonsymptomatic leaves from diseased plants transpired one-third as rapidly as leaves from healthy plants supplied with the same amount of water. Drought-hardened leaves transpired one-sixth as rapidly as succulent healthy leaves [Dimond & Waggoner (27)].

The stomatal behavior of leaves on healthy and diseased plants aids in interpreting their transpirational pattern. In tomato wilt the water loss from diseased plants is slightly less than from healthy ones for the first few days after inoculation, then becomes slightly greater than that of the healthy plants until the first symptoms of water shortage appear [Scheffer & Walker (58), Ludwig (24)]. Once this happens, diseased plants transpire considerably less than do healthy ones (24, 27, 58).

Effects of Disease on the Permeability of Leaf Cells

The effect of wilt diseases on the osmotic mechanism of leaf cells is not well-known. *Verticillium* infection causes a rapid wilting of leaves when it invades the foliar spongy parenchyma [Van der Lek (59)]. In this study, however, no correlation was made between the onset of wilting and the presence of mycelium. Other leaf-invading fungi such as the rusts, the powdery mildews, and the apple scab fungus do not cause wilting. The *Fusaria* apparently do not invade leaf parenchyma but cause wilting in their hosts.

Culture filtrates of *F. oxysporum f. lycopersici* apparently increased the permeability of leaf cells [Thatcher (60)]. Such measurements are difficult to evaluate because, of the many phytotoxic components of culture filtrates, none have been demonstrated to function in pathogenesis [Dimond & Waggoner (61)].

Cells of diseased plants may be more permeable than those of healthy ones. Thus, exposure of parenchymatous cells to the tracheal sap from diseased tomato plants increased the time for them to recover their turgor

after plasmolysis, whereas the sap from drought-wilted plants had little effect. Although this observation suggests a greater permeability in cells of diseased plants, there is no suggestion of destroyed osmotic function and the effect is reversible [Gottlieb (36)]. We know today that the tracheal fluid of diseased, but not of healthy plants, contains polyphenols which are thought to lead to vascular discoloration [Davis, Waggoner & Dimond (62)]. A plant which has absorbed gallic acid wilts on a sunny day but recovers at night [Davis & Dimond (63)]. Such compounds may be responsible for the effects observed by Gottlieb (36). Increased permeability of leaf cells may cause the increase in transpiration from leaves before diseased plants become symptomatic.

Whether or not leaf cells of diseased plants have a permeability to water which is chronically higher than in healthy plants, their osmotic mechanism continues to function, even in severely wilted (but not withered) leaves. Severely wilted leaves from *Fusarium*-infected plants recover their turgor if they are immersed in water. The vascular bundles in petioles of such leaves are strongly discolored and evidently are dysfunctional in water transport. If the base of such a petiole is placed under water, the leaflet does not recover its turgor, but if the discolored portion of the petiole is cut off and the remaining stub is placed in water, the leaflet recovers promptly [Dimond & Waggoner (27)].

The Role of Toxins in Wilt Pathogenesis

Wilt-inducing pathogens have long been thought to produce toxins which are liberated into the host and translocated to leaf cells, destroying their differential permeability and thus causing wilting [Hutchinson (64)]. Today, the toxin theory has been extended to cover any material, arising either from the pathogen or its action upon the host, which impairs the normal functioning of the host. The toxin theory is attractive because it offers an explanation for the development of disease symptoms in advance of the pathogen, e.g., vascular discoloration in *Verticillium* wilt of mint (12). Moreover, when a metabolic product of the pathogen produces symptoms in cuttings which resemble disease symptoms, it is tempting to seize upon this resemblance. For these reasons, the toxin theory has been widely accepted. However this may be, the discussion of the theory will be restricted here to the compounds suggested as toxins and the role they may play in wilt pathogenesis, excluding materials which lead to water shortage by plugging vessels.

Experimentally, the search for toxins in culture filtrates of wilt-inducing pathogens has frequently been based upon two assumptions: (*a*) that wilting of cuttings is a sufficiently specific response to be useful, and (*b*) that metabolic products of the pathogen formed *in vitro* are also formed in diseased plants and function in pathogenesis.

Both assumptions are dangerous extrapolations. The wilting of a plant shoot is by no means a specific response. Almost any large molecule inter-

feres with water transport in a physical way and causes cuttings to wilt [Hodgson, Peterson & Riker (65)]. Pectins and other polysaccharides, proteins [Thornberry & Ray (66)], the debris of cells resulting from the maceration of the end of the cutting by enzymes in the filtrate, even bacteria which may subsequently grow in the test solution—all produce wilting of test cuttings. Purified culture filtrates may contain sufficient enzyme protein to cause plugging nonspecifically. Such responses may or may not bear upon what happens in diseased plants. Hursh has urged a more critical examination of the cause of water shortage in diseased plants and the use of these findings in examining compounds thought to be responsible for wilting in such plants (50).

Specific criteria are needed to establish the role of a compound in producing symptoms in diseased plants. Three criteria have been proposed: the compound must be (*a*) isolated from the diseased but not be present in the healthy host; (*b*) characterized chemically; and (*c*) reintroduced in pure form into a healthy host where it must produce the symptoms of disease or a portion of the syndrome. Such a compound is called a vivotoxin to distinguish it from the toxic compound isolated from culture filtrates but not demonstrated to have a role in pathogenesis of the infected plant [Dimond & Waggoner (61)]. Where two or more enzymes as a team are involved in pathogenesis, as may be the case in *Fusarium* wilt of tomato, these criteria must be modified accordingly.

Toxins from culture filtrates.—Many compounds have been found in culture filtrates which are phytotoxic but none of the simple compounds appears to be the cause of wilting in infected plants. The extensive literature prior to 1946 has been reviewed adequately (13, 36, 67, 68, 69). As yet, ethylene is the only compound produced in *Fusarium* culture filtrates which has been demonstrated to be a vivotoxin [Dimond & Waggoner (14)].

The development of penicillin and other antibiotics has focussed attention on the biologically active compounds produced by microorganisms. Since 1944, a number of compounds have been isolated and identified from cultures of plant pathogens. Some of the compounds produce phytotoxic symptoms in plants which more or less resemble the disease symptoms found in plants infected by the source pathogen. A considerable literature has been built up around these compounds, their biological properties, and their role in pathogenesis. Because these compounds have been widely discussed as pathogenic agents in wilt diseases, the work will be reviewed in considerable detail.

F. oxysporum f. lycopersici produces lycomarasmin and fusarinic acid in culture [Clauson-Kaas *et al.* (70); Gaumann & Naef-Roth (71); Gaumann *et al.* (72)]. Because of their action on tomato shoots these compounds are assumed to have a role in disease production *in vivo* [Gaumann *et al.* (72); Gaumann (73 to 76)]. In addition, vasofuscarin, an unidentified fraction from cultures of the tomato wilt *Fusarium*, causes a vascular browning of cuttings [Gaumann, Stoll & Kern (77)]. Together with alternaric acid, which is pro-

duced in culture by *Alternaria solani* [Brian *et al.* (78)], such compounds have been called wilting toxins[4] (73 to 76).

Lycomarasmin, the first of the wilting toxins to be isolated, can be purified from six- to eight-week cultures of the tomato wilt *Fusarium*. It is a dipeptide, having a molecular weight of 277 and an empirical formula of $C_9H_{15}O_7N_3$ [Plattner & Clauson-Kaas (79)]. Products of hydrolysis are glycine, aspartic acid, ammonia, and pyruvic acid [Plattner & Clauson-Kaas (80); Plattner *et al.* (81)]. From this and other evidence, Woolley proposed lycomarasmin to be α-hydroxy-α-acetaminopropionyl-glycylasparagine, synthesized this compound and showed its toxicity to be comparable qualitatively and quantitatively to the natural product (82). A number of related synthetic compounds were less active [Woolley (83)]. On heating or long standing in aqueous solution, one mole of NH_3 is liberated per mole of lycomarasmin, the remaining breakdown product being nontoxic (79). Decomposition probably involves hydrolysis of the asparagine amide group of the lycomarasmin molecule [Woolley (82)].

When absorbed by tomato cuttings, lycomarasmin causes the margins of leaflets to roll upwards and become brittle and withered [Clauson-Kaas *et al.* (70)]. It does not cause a true wilting and petioles do not flag. [See illustrations in Gaumann & Jaag (84)]. A necrotic spotting develops in interveinal areas of leaflets under certain conditions [Gaumann *et al.* (85)]. Toxicity is light dependent [Gaumann (74); Gaumann & Jaag (84)].

Lycomarasmin, other wilting toxins, and certain antibiotics which cause leaf spotting and marginal withering of leaves all affect the water economy of plant shoots in a characteristic way. In a controlled environment, both the transpiration and absorption of water drop rapidly and equally to one-third of the initial values when tomato cuttings absorb 0.01 *M* lycomarasmin. This is the shock phase of lycomarasmin response. Then both transpiration and absorption increase over the next 3 or 4 hr., transpiration being higher and absorption lower than initial values. This is the phase of excessive transpiration and results in loss of fresh weight of the cutting. At this time the first symptoms of desiccation appear. Thereafter, transpiration, water absorption, and fresh weight all decrease and withering becomes pronounced. This is the phase of paralysis. More dilute solutions alter the intensity but not the course of the response. [Gaumann & Jaag (84, 86, 87); Gaumann, Naef-Roth & Kobel (72); Gaumann, Kern & Sauthoff (85)].

The action of lycomarasmin on tomato tissue has been interpreted on a cellular basis [Gaumann (74); Gaumann & Jaag (84, 86)]. During the shock phase the wilting toxin is said to become distributed throughout the cutting,

[4] A wilting toxin, according to Gaumann's usage (74, 75), is a compound produced by a pathogen which causes irreversible changes in the water economy of plant tissue. These changes may express themselves as wilting, withering, stiffening, necrotic spotting, or as other symptoms of phytotoxicity. In this usage, wilting refers to a condition in which cells may be partially or completely dehydrated and in which they may be living or dead. In the present paper, wilting refers to simple loss of turgor.

just as fluorescent compounds of comparable molecular weight are (84, 86). Lycomarasmin is considered to bring on the phase of excessive transpiration by coagulating protoplasm. This causes a release of water from cells which is then transpired without a corresponding increase in uptake. Such an interpretation is supported by the observation that lycomarasmin causes protoplasmic streaming to cease in *Tradescantia* leaf hairs in 30 min. and coagulates the protoplasmin within 4 hr. (74). However, lycomarasmin is very toxic to some organisms and without effect on others [Gaumann *et al.* (88, 89)]. The release of water from cells in itself is said not to damage them. Rather, cells are damaged during the phase of paralysis when differential permeability of leaf cells is considered to be destroyed. Tomato leaf cells are known to increase in permeability when exposed to crude *Fusarium*-culture filtrates or to lycomarasmin [Thatcher (60); Gaumann *et al.* (88)]. The visible phytotoxicity of lycomarasmin is irreversible (84, 86).

Three types of wilting[5] have been differentiated [Gaumann (74, 75); Gaumann & Jaag (86, 87, 90)]. Physiological wilting results from simple water shortage, is reversible when water loss is not excessive and, if irreversible, the toxic effect results from water loss alone. In physiological wilting a plant shoot absorbs less water than it transpires. This results in a loss in fresh weight. Typical pathological wilting[5] results from the action of the wilting toxins of plant pathogens and is characterized by three phases of response: the phases of shock, excessive transpiration, and paralysis. Physical wilting,[5] a type of pathological wilting, is said to result from the physical action of polysaccharides or other large molecules in intracellular spaces of the leaf. When large molecules plug the stem only, the leaf shows physiological wilting. In physical wilting the water uptake and transpiration both show a steady decrease to as little as 10 per cent of initial rates, although fresh weight is unchanged [Gaumann (74); Gaumann & Jaag (90)]. Inulin, a polysaccharide causing physical wilting, produces a withering and crisp drying of leaflet margins which resembles that produced by lycomarasmin (90).

The concept of what constitutes pathological wilting is elusive. Physiological and pathological wilting can be distinguished only in terms of how water uptake and transpiration vary with time, relative to the initial rate and to one another and not in terms of loss in fresh weight alone. When a cutting responds to high concentrations of a wilting toxin, pathological wilting is characterized by all three phases of response, including the phase of paralysis which indicates irreversible destruction of the differential permeability of leaf cells [Gaumann (74, 75); Gaumann & Jaag (86, 87, 90)]. However, when a cutting responds to low concentrations of a wilting toxin, the phase of paralysis may not appear. Cuttings absorbing 10^{-5} *M* lycomarasmin develop no phytotoxic symptoms but show a shock phase. The rate of

[5] The terms physical and pathological wilting as used by Gaumann do not necessarily refer to loss of turgor. See footnote 4.

transpiration then increases but remains less than the initial rate. Gaumann suggests that this response illustrates pathological water loss without wilting, i.e., Gaumann's usage of the term wilting.[5] Cuttings exposed to 0.01 *M* lycomarasmin in a saturated atmosphere develop characteristic phytotoxic symptoms and illustrate pathological wilting without water loss (73, 74). In this case the loss in fresh weight is no more than 3 per cent of the initial weight. Apparently, the important characteristic of pathological wilting is that it is a chemically-induced loss in differential permeability of leaf cells, resulting in a phase of paralysis.

From such evidence it appears that wilting toxins have a specific action on cellular membranes. Perhaps this feature has been overemphasized. What is important is that the cell is injured by the wilting toxins. We cannot say today whether wilting toxins are specific membrane poisons or whether they poison cells and thus lead to loss of differential permeability.

Lycomarasmin forms a colorless water-soluble complex with iron which is more toxic to tomato shoots than lycomarasmin alone [Plattner & Clauson-Kaas (79)]. With suitable adjustment for its greater toxicity, the effect of the lycomarasmin-iron complex on the plant is the same in all respects as for lycomarasmin alone, except that brown flecking of interveinal areas of the leaf is more prominent in this case [Gaumann, Kern & Sauthoff (85)].

The minimum toxic dose of pure lycomarasmin is 150 mg./kg. fresh weight of tomato tissue and that of the lycomarasmin-iron complex is 15 mg./kg. This index is useful for comparing the sensitivity of tomato varieties to lycomarasmin. From the immune Red Currant variety to the highly susceptible Bonny Best, all varieties have the same minimum toxic dose [Gaumann (74); Gaumann, Naef-Roth & Meischer (91)]. The highly virulent R-5-6 strain of *F. oxysporum f. lycopersici* produces less lycomarasmin than one of low virulence to tomatoes (74, 91).

Lycomarasmin has another interesting biological property. On *Lactobacillus casei*, it reverses the action of strepogenin, a growth factor of unknown constitution, and strepogenin reverses the action of lycomarasmin on tomato cuttings [Woolley (92); Gaumann *et al.* (93)]. Because concentrates high in strepogenin activity are rich in glutamic acid and lycomarasmin contains asparagine, Woolley explored the possibility that lycomarasmin might be structurally related to strepogenin (92). This line of reasoning prompted Albert to suggest that only the high cost of glutathione prevented its use in control of *Fusarium* wilt of tomato (94). In point of fact, the lycomarasmin-iron complex in the presence of glutamine, glutamic acid, glytathione, or cysteine is at least as toxic to tomato cuttings as is the lycomarasmin-iron complex alone [Dimond & Waggoner (95)].

Lycomarasmin has been said to cause the wilting in *Fusarium* wilt of tomato [Gaumann (73, 74, 75)]. But neither it nor fusarinic acid, a second wilting toxin of the tomato wilt *Fusarium*, has ever been detected in or isolated from diseased plants. The symptoms of lycomarasmin and fusarinic acid toxicity bear no more than a superficial resemblance to the foliar symptoms

of *Fusarium* wilt. Foliage does not recover from lycomarasmin-induced symptoms, but wilting leaves on diseased plants recover their turgor when they can obtain water [Dimond & Waggoner (27)]. If lycomarasmin plays a role in the syndrome of *Fusarium* wilt of tomatoes, symptoms on infected iron-deficient plants should be less severe than on normal infected plants. This is not the case [Scheffer & Walker (42)]. There is no evidence in the transpirational history of an infected plant of a shock phase and a closely following phase of excessive transpiration which is characteristic of the action of lycomarasmin and fusarinic acid (42).

If lycomarasmin is produced immediately in infected plants at the rate at which it appears in cultures after six weeks, the amount that would be formed in the normal incubation period of the disease is much less than that required to cause wilting [Scheffer (96)]. Because lycomarasmin breaks down at a measureable rate at 25°C., the rate of actual production is higher than the rate of its appearance in culture. If host cells are assumed to accumulate lycomarasmin as it is produced so that breakdown does not occur, estimates of production in the plant must be corrected for the amount disappearing from culture filtrates through decomposition. Through measuring the NH_3 liberated as lycomarasmin breaks down, the rate of decomposition at 25°C. has been measured. When estimates are corrected to obtain the true production rate, lycomarasmin is found to be produced at one-eighth of the rate necessary to account for wilt production in diseased plants [Dimond & Waggoner (97)].

Studies on lycomarasmin production have shown it to appear only after lysis of mycelium begins, and not in cultures known to be free of lysing mycelium [Dimond & Waggoner (49)]. If it is a product of lysing mycelium, it must be liberated in the infected plant from the oldest mycelium, located at invasion points in the roots. According to Gaumann & Jaag, lycomarasmin becomes systemically distributed in the plant (84, 86). This should cause systemic and simultaneous symptoms to develop on all fully formed leaves. But the symptoms of *Fusarium* wilt develop characteristically on oldest leaves first and progress in an orderly fashion to successively younger ones.

Lycomarasmin forms a colorless complex with iron and a blue complex with copper ion. Only the iron complex is toxic to plants [Plattner & Clauson-Kaas (79)]. The metal-binding properties are a result of chelation, as suggested by its chemical structure and shown by two lines of evidence. Titration curves show hydrogen ions to be liberated from the molecule as chelation occurs [Waggoner & Dimond (98)]. Also, when lycomarasmin breaks down on heating, it simultaneously loses biological activity and chelating ability [Dimond & Waggoner (97)]. These observations suggest the participation in chelate bonding of a carboxyl group and of the N atom split from the lycomarasmin on decomposition. These groups are in close proximity in the asparagine moiety of the molecule [Waggoner & Dimond (98)]. Other wilting toxins also form chelate complexes, e.g., fusarinic acid [Deuel (99)].

If wilting toxins are chelating agents, they may be toxic by virtue of

forming chelate complexes in the plant, thus robbing it of essential trace elements [Deuel (99); Gaumann & Naef-Roth (100)]. In support of this interpretation is the fact that ethylene diamine tetraacetic acid, a well-known chelating agent, produces symptoms in tomatoes which resemble those produced by lycomarasmin and fusarinic acid (100).

Two lines of evidence suggest that this thesis may be questioned. First, lycomarasmin forms chelate complexes both with iron and copper ions. The copper complex is not toxic but is more stable than the iron complex. In the presence of copper ion, the iron complex is rendered nontoxic to tomato cuttings if lycomarasmin concentrations are limiting in the system for chelation but are at phytotoxic levels [Waggoner & Dimond (98)]. Second, the iron complex, preformed before entering the plant, is more toxic than lycomarasmin alone [Gaumann (74); Gaumann *et al.* (85)]. This relation can be tested in another way. A synthetic chelating agent such as 8-quinolinol, which forms an iron complex of greater stability than that of lycomarasmin, will rob the iron from lycomarasmin. When iron is limiting in the system, the addition of 8-quinolinol to the lycomarasmin-iron complex renders lycomarasmin quite nontoxic to tomato cuttings, even when lycomarasmin, in sufficient quantity to be toxic, is present alone. Apparently lycomarasmin is toxic only as the iron complex and not as the free molecule [Waggoner & Dimond (98)]. On this basis pure lycomarasmin, when introduced into the plant, becomes toxic only after it robs the plant of sufficient iron to form the complex. Simple calculations, based on the minimum toxic dose of lycomarasmin, 150 mg./kg. fresh weight of tomato tissue, indicate that 0.27 millimols of iron per kg. would be removed from the plant, assuming that, in chelation, the molar ratio of lycomarasmin to iron is 2:1. Whether or not this reduction in iron content would produce an iron deficiency in the plant is problematical.

There are parallels for the argument that the iron complex is what is toxic rather than the unchelated molecule. One of the roles of trace elements in the cell is the formation of chelate complexes by specific elements which are biologically active only as the complex. Chlorophyll and cytochrome are but two of many examples which could be given.

The chelate properties of lycomarasmin can be used to test for the presence of this molecule in *Fusarium*-infected tomato plants. If the presence of either copper ion or 8-quinolinol renders the lycomarasmin-iron complex nontoxic, their introduction into infected plants should reduce the severity of wilting symptoms induced by lycomarasmin. Both $CuSO_4$ and 8-quinolinol are absorbed by tomato plants. When infected plants, scored for severity of wilting symptoms at the beginning of the experiment were treated with $CuSO_4$ or 8-quinolinol, the increase in severity of wilting symptoms after several days was the same as for plants receiving no treatment. Thus, lycomarasmin was not detected by this technique (98).

In summary, lycomarasmin and fusarinic acid are produced in cultures and are phytotoxic [Gaumann (74); Gaumann, Naef-Roth & Kobel (71, 72)]. Until their presence in diseased plants has been clearly demonstrated,

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their role in the syndrome of the infected plant must remain in question [Dimond & Waggoner (61)]. Also the present picture of their action in plants must be modified to account for the progressive symptom development of *Fusarium* in infected plants. Vasofuscarin, another wilting toxin in culture filtrates of the tomato wilt *Fusarium*, and also claimed to play a role in disease development, must similarly be shown to fulfill the criteria of vivotoxicity [Gaumann, Stoll & Kern (77); Dimond & Waggoner (61)].

Toxins from diseased plants.—Only one series of experiments has dealt with toxic materials which occur in infected plants. This is the work of Gottlieb, who centrifuged the tracheal fluid out of infected and healthy stem sections. The resulting fluid from infected plants caused wilting of tomato cuttings whereas that from healthy or from physiologically wilted plants did not (36, 101). The wilting so induced was reversible and if transpiration of cuttings exposed to this material was prevented, no wilting occurred. This material increased the permeability of tomato cells (36). It was heat stable, could be distilled under reduced pressure at 95°C., was adsorbed on activated charcoal, and was stable in the presence of O_2. Spectrographic analysis revealed no differences between tracheal fluids from healthy or infected plants (36). The nature of this material and how it arises is still unknown. Because its effect on permeability of cells is reversible it is probably not lycomarasmin. Gottlieb has suggested that it is as likely to arise from injured host cells as from the fungus itself (101).

EVIDENCE ON THE CAUSE OF VASCULAR DISCOLORATION

Suggestions as to the cause of vascular discoloration have been few. Vascular discoloration is probably not the result of a single chemical reaction, but is more probably the result of two or more independent processes.

The mycelium of *Verticillium* becomes black as it ages and in the *Verticillium* wilts, old mycelium in vessels probably contributes to the dark color of vascular bundles [Rudolph (17)]. The mycelium of *Fusarium*, however, remains hyaline, even when it is old.

Dark colored gums in vessels may contribute substantially to vascular discoloration, e.g., in oak wilt [Struckmeyer *et al.* (43)]. In other wilt diseases gum occurs in some vessels in late stages of disease after wilting has appeared. It is preceded in appearance by a grey homogeneous occluding material. The latter disappears when sections are fixed and stained but in living material it gradually becomes dark brown and granular [Ludwig (24); Bewley (45)]. Probably the light colored material contributes to water shortage in leaves by occluding vessels, but only as it becomes dark colored does it contribute to vascular discoloration. By this time vascular discoloration from other causes is already well developed.

In early stages of disease, discoloration can be seen to be limited to living xylem cells. Careful dissections reveal that even the walls of xylem vessels adjacent to such cells are not discolored. This aspect of vascular discoloration may be quite separate from that due to dark colored gums.

Discoloration of living xylem cells may result from the oxidation of phenols to produce melanoid pigments and these pigments may eventually become trapped in occluding gums in vessels. When adsorbed by tomato cuttings a number of polyphenols, e.g., pyrogallol and gallic acid, produce discoloration of xylem parenchyma in a pattern indistinguishable from that present in *Fusarium*-infected plants [Davis, Waggoner & Dimond (62)]. Dilute solutions of browning phenols produce discoloration only for a short distance from their point of entry; concentrated solutions (1000 p.p.m.) cause a discoloration which extends into petioles and the growing point [Davis & Dimond (63)]. In this respect also the discoloration produced by dilute solutions of phenolic compounds resembles that produced by *Fusarium* because the latter ordinarily occurs only slightly in advance of mycelium in the host [Dimond *et al.* (15)]. When discoloration occurs well in advance of mycelium, as in *Verticillium* wilt of mint (12), production of larger amounts of phenol may occur.

Tracheal fluid of diseased but not of healthy tomato plants contains phenolic substances as indicated by the Folin Ciocalteu reagent or by melanin formation on addition of mushroom tyrosinase [Davis, Waggoner & Dimond (62)]. These responses suggest the oxidation of suitable polyphenols by terminal oxidases in living xylem cells. Evidence for the presence of polyphenolases in tomato tissue has been presented by Link, Klein & Guzman Barron (102).

The polyphenols themselves may arise in diseased plants in two ways. Infected tomato plants contain fewer lignified cells and these cells have less lignin in their walls than cells in healthy plants as shown by the phloroglucinol-HCl reagent [Davis & Dimond (63)]. Freudenberg has proposed a mechanism for lignin synthesis which depends upon the action of β-glucosidase on specific phenolic glycosides to liberate phenols. These phenols are then oxidized in lignin formation (103). In diseased plants, the phenols may be oxidized in a different manner by terminal oxidases, forming melanins instead of lignin. These dark colored pigments could cause vascular discoloration (62).

Alternatively, polyphenols can arise through hydrolysis of a wide range of phenolic glycosides of the host. The hydrolyzing enzymes, β-glucosidases, are already present in host cells and have been demonstrated to be produced by the tomato wilt *Fusarium*, both *in vitro* and in the host. As host cells become disorganized through action of the pathogen, a chain of reactions is thought to occur. Glycosides are hydrolyzed, phenols are liberated, and these move to living cells where they are oxidized to discoloring melanins [Davis, Waggoner & Dimond (62)].

Vascular discoloration has also been attributed to the action of pectin methyl esterase (PME).[2] A few hours after mycelial mats of *F. oxysporum f. lycopersici* are placed on fresh nutrient, the culture filtrate contains a material which causes vascular discoloration in tomato cuttings. This material is nondialyzable and is precipitated by alcohol [Scheffer & Walker (42)]. A

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number of commercial pectic enzyme preparations and water extracts of *Fusarium* cultures on bran contain amounts of PME which are correlated with the intensity of the discoloration these preparations produce in tomato stems. [Gothoskar *et al.* (52)]. Other wilt and root rot-producing pathogens also produced this enzyme [Winstead & Walker (53)]. How PME could be responsible for the browning reactions or for plugging is not clear. The extent of discoloration is unrelated to the amount of polygalacturonase (PG)[2] present (52, 53). However, the amount of vascular discoloration produced in tomato stems is not necessarily related to the amount of PME in preparations which cuttings absorb. When *Fusarium* is grown on pectin, the amount of PME is ten times greater than in bran cultures, but the amount of discoloration is the same. Moreover, when *Fusarium* PME is concentrated and purified by elution from Celite with NaCl, its ability to cause discoloration is not great. In all preparations examined, whether commercial enzyme preparations or from *Fusarium* cultures, β-glucosidase activity is present [Waggoner & Dimond (51)]. Remembering that the thesis of vascular browning by PME is based on studies in which unpurified preparations were employed and considering the possible role which β-glucosidases may play in vascular discoloration, one cannot consider the action of PME in producing discoloration and wilting as established.

Vasofuscarin, a fraction isolated from culture filtrates of the tomato wilt *Fusarium*, causes browning of vascular bundles or cuttings and is claimed to be the cause of discoloration in tomato wilt [Gaumann, Stoll & Kern (77)]. Its chemical nature, the mechanism by which it causes discoloration, and its role in diseased plants are all unknown. Vasofuscarin is believed to be an enzyme (77) and may prove to be PME or a β-glucosidase.

MECHANISMS TO ACCOUNT FOR WILTING AND DISCOLORATION

Pectic compounds have been suspected as a cause of plugging in xylem vessels and hence of wilting (24, 50). Gums which occlude vessels may have been converted from host pectic materials. Pectic enzymes, both PG and PME, are necessary for these conversions. Their action on middle lamella, exposed at pits in vessels, might result in random fission of pectin to produce pectinic acid, which could form Ca gels [Waggoner & Dimond (51)]. Alternatively, the products of complete pectin hydrolysis might be converted to gums [Higgins (54)].

PG is not known in tomato plants [Kertesz (104)]. For pectin hydrolysis, PG of fungal origin is necessary. The tomato wilt *Fusarium* produces both PG and PME in abundance *in vitro* on a pectin substrate. PG is formed rapidly and PME is formed slowly on a glucose medium. PME of fungal origin has been detected in tracheal fluid from diseased plants, although PME from the host is also present [Waggoner & Dimond (51, 105)]. These enzymes may be functional in producing wilt.

They may also function in producing vascular discoloration. Unless some

maceration of xylem parenchyma occurs, β-glucosidase from the fungus cannot encounter its substrate in living cells (51, 62). Unless living cells are disorganized by such injury as maceration, host β-glucosidase in these cells will not hydrolyze its substrate (51, 63). Both PG and PME are necessary for maceration to occur.

In view of the necessity of the presence of PG for maceration, it is difficult to see how PME alone can be involved as a cause of both discoloration and wilting, as proposed by Gothosakar *et al.* and by Winstead & Walker (52, 53).

A small amount of maceration, brought on by PG and PME together, could trigger the whole mechanism for wilting and discoloration. Products of maceration could cause plugging and wilting. Products of glycoside hydrolysis could cause vascular discoloration by the chain of reactions already outlined (51, 62, 63). Finally, polyphenols themselves may be a cause of wilting (63).

WILT PATHOGENESIS: A SYNTHESIS

The syndromes of the many vascular wilt diseases have many features in common and probably have a common biochemical basis. The principal symptoms consist of epinasty, vascular discoloration and wilting, with attendant desiccation. To account for the variation in the syndromes across the range of diseases, it is suggested that one pathogen or one host may lack a component which prevents that symptom from developing. Epinasty and leaf yellowing appear to be caused by ethylene formation by the pathogen and possibly to some extent by the host. The ethylene is liberated into the transpiration stream, becomes systemically distributed, and causes epinasty on all leaves before other conspicuous symptoms develop. Some wilt-inducing pathogens may produce more ethylene than others.

Vascular discoloration appears to result from melanin production in living cells. Melanin is produced through the oxidation of polyphenols by terminal oxidases. The polyphenols appear to arise from hydrolysis of glycosides by β-glucosidase. β-glucosidase in the host cell or that produced by the pathogen may be involved. In either case the reaction may be initiated through disorganization of cells, brought on by the action of fungal pectic enzymes. In the case of hosts which lack such oxidases in xylem parenchyma or pathogens which do not form a β-glucosidase, no discoloration develops, e.g., watermelon wilt.

The wilt-producing pathogens produce pectic enzymes which partially or completely hydrolyze pectins exposed at pits in vessels. The hydrolytic products can form calcium gels if partially hydrolyzed or gums if acted upon in turn by other enzymes. In either case, plugging of vessels and water shortage in leaves develops and becomes more acute with time. As melanoid pigments are released into the vascular elements, the pigment may be trapped by the gels, contributing to vascular discoloration. Alternatively, the gums

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may polymerize as they age and become darker in color. Vascular discoloration may thus result from more than one process.

All of these reactions proceed in the vicinity of the mycelium itself and thus lead to progressive symptom development. In some cases, mycelium forms conidia in vessels which become dislodged and are moved through vessels with the transpiration stream. These conidia germinate, and if many are involved, produce a systemic infection in the plant. Then, by repetition of the above processes on a systemic scale, the entire plant collapses at once.

Present thinking suggests that no one toxin or enzyme will account for the syndrome of any one wilt disease. Rather a number of mechanisms are involved. An understanding of these mechanisms is necessary in developing control measures for the wilt diseases which are based on blocking symptom development.

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CHEMICAL NATURE OF DISEASE RESISTANCE IN PLANTS[1,2]

By J. C. Walker and M. A. Stahmann

Departments of Plant Pathology and Biochemistry, University of Wisconsin, Madison, Wisconsin

For the past half century there has been a steady increase in interest and research on the part of plant pathologists in the use of host plant resistance as a means of disease control. Before 1900 only an occasional crop variety had recognized merit as resistant to one or more important diseases. At present it would not be difficult to list some hundreds of such varieties in the United States alone, and each year brings additional ones into use. While induced immunity is the type considered most important and is generally relied upon in human and animal pathology it is not made use of at all in plant pathology in so far as disease control is concerned. The production of so-called antibodies in response to infection has not been clearly demonstrated in plant tissue. In plants, as in animals, however, it is generally observed that within a variety or species individuals often occur which are naturally more resistant than the remaining population to a given disease. During epidemics such plants stand out most conspicuously and in times past selection has gone on in nature or has been made empirically by cultivators leading, in some cases, to "naturally" resistant varieties. Soon after the rediscovery of Mendel's law in 1900, Biffen (4) suggested that the natural resistance of a variety of wheat to yellow rust [*Puccinia glumarum* (Schm.) Eriks. and E. Henn.] was inherited as a Mendelian character. Since that time dozens of cases of resistance have been shown to be inherited as unit characters, others as multigenic characters. It has also been shown, as in many other Mendelian characters, that the phenotypic expression of inherited resistance may be suppressed or enhanced by certain combinations of environment to which the diseased plant is exposed. When it became generally recognized that many cases of plant resistance are gene-controlled, this process of disease control was greatly stimulated through development of resistant varieties in which resistance to one or another disease or to several diseases was transferred by breeding procedures to nonresistant but otherwise desirable agricultural varieties.

The nature of resistant characters, aside from their mode of inheritance, has been a subject of some interest since about 1890. Cobb (8) pointed out certain morphological differences in varieties of wheat which were resistant and susceptible, respectively, to the stem-rust fungus., *P. graminis* Pers. Following this, numerous cases have been described in which the resistant

[1] The survey of the literature pertaining to this review was completed in December, 1954.

[2] The following abbreviations will be used: Pectin methyl esterase, PME; Polygalacturonase, PG; Depolymerase, DP.

character was associated with one or another morphological or histological character of the plant, a condition which was less pronounced or absent in susceptible plants. Many histological studies showed, on the other hand, that resistance was not generally associated with any histological difference between varieties and this pointed more directly to biochemical or physiological differences which might explain in part, at least, the basis of resistance. The literature up to 1924 has been reviewed by Walker (48).

Penetration of the host by fungus and bacterial pathogens is the first step in host-parasite interaction which may lead to established infection and consequent disease development. It is possible for the resistant character to be one of exclusion of the potential pathogen or, as is more often the case, the resistant character is one restricting establishment of infection or in slowing down disease development. Penetration takes place in one of three channels. The first of these is direct penetration in which the cuticular surface of leaf or stem is penetrated, presumably by mechanical pressure, and without the aid of enzymatic secretion; in some cases of noncuticularized surfaces direct penetration may be accomplished by enzymic digestion of cell membranes. In the second type of penetration invasion is by way of natural openings, notably stomata, hydothodes, or lenticles. In the third type, natural or inflicted wounds are channels of penetration, e.g., natural wounds of root cortex owing to rupture by secondary roots, insect wounds, mechanical abrasions caused by wind-blown dust, hail, and by harvesting procedures. The method of penetration is an inherent character of the pathogen influenced little by the host. In some pathogens, notably the rust fungi, the type of invasion is obviously gene-controlled. In the rusts in the genera *Puccinia* and *Uromyces* uninucleate, monokaryotic mycelium penetrates the host directly, while the binucleate, dicaryotic stage always penetrates through stomata. This holds whether or not in the rust species concerned the two stages penetrate the same or distinct host species. Several fungi have been shown to penetrate a large number of nonhost species. It is interesting to note that in each such case the type of penetration of the nonhost is exactly the same as that of the host species. Arens (1) showed that the downy mildew pathogen, *Plasmopara viticola* (Berk. and Curt.), Berk. and DeT., of grape penetrates the stomata of many species plant including the grape but it establishes infection only in the grape and certain closely related species. The onion smudge organism [*Colletotrichum circinans* (Berk.) Vogl.] penetrates onion, onion relatives, and a number of unrelated species directly but it usually becomes established only in the onion and closely related species [Johnson (26)]. When resistant and susceptible varieties of the same host species are compared, in most, but not all cases, penetration of the resistant host takes place in the same manner as in the susceptible host. The resistant character is usually expressed as infection is becoming established or during later development of disease. It may be said, therefore, that, in general, the mode of penetration is determined by an inherent character of the pathogen and that it is, in most cases, little influenced by the host sub-

strate. Whether or not a penetrating fungus or bacterium becomes a pathogen and what degree of pathogenicity it develops is determined largely during the interaction of host and parasite.

We may now consider some specific cases in which the chemical nature of the resistant character has received detailed study. We shall not be concerned with the numerous cases in which the degree of parasitism is influenced by the nature of the host substrate, but more particularly with those cases in which there are distinct differences between varieties in their resistance to a given pathogen.

Cook *et al.* (9) early pointed out the possibility that at the point of penetration the activity of polyphenol oxidases might release phenols into the substrate which inhibit progress of the pathogen. Newton *et al.* (31, 32) found that the wheat variety Khapli resistant to stem rust (*P. graminis* Pers.) was higher in phenol content than the very susceptible varieties Marquis and Little Club. The most extensively studied case is that of onion smudge. This is a disease which is concerned primarily with the fleshy bulb [Walker (46)]. The pathogen *C. circinans* [(Berk.) Vogl.], is a soil fungus which appears to subsist successfully as a saprophyte and has little tendency to attack actively growing parts of the plant. Its relation to the host begins in a saprophytic development on the thin outer scale and contiguous basal portions of the leaf which consist largely of dead tissue. Once established on its host the fungus penetrates underlying fleshy scales, proceeding slowly to promote a gradual shrinkage of the tissue and to penetrate successively the underlying scales. This fungus, like all species of *Colletotrichum* studied in this regard, penetrates the host cuticle directly and mechanically after having formed at the contact of host surface and hyphal tip a distinct thick- and dark-walled appressorium surrounded by a gelatinous matrix which serves to cement that organ to the host. Once past the thin layer of cutin the fungus mycelium becomes established first between the cuticle and the subcuticular outer cellulose wall of the epidermal cell, lifting the relatively stretchable cutin layer as more room is required for the fungus. In contrast to the purely mechanical stretching effect on the cuticle, the fungus, through the secretion of one or more exoenzymes, causes a slow digestion of the relatively thick subcuticular wall. By such action the protoplasm of the epidermal cell is so affected as to show signs of degeneration even before the mycelium of the pathogen has invaded the cell lumen.

The high resistance of onion varieties in which the bulb scales are pigmented, red or yellow, is well known [Walker (47); Walker & Lindegren (54)]. This resistant character has been shown to be controlled by the interaction of three gene pairs [Rieman (37); Clarke *et al.* (7); Jones *et al.* (25)]. A close association of resistance with bulb pigment is evident even in colored bulbs where in the absence or dilution of pigment near the neck of the bulb, infection commonly takes place. The onion bulb as it matures has one to three outer dry scales consisting of dead tissue which are, in colored varieties, the most highly pigmented. In the underlying scales the pigment is found only

in the cell sap of the outer epidermal cells. Yellow flavone pigments which are derivatives of quercetin are found in both red and yellow varieties, probably as glycosides, and in the dry outer scales quercetin occurs in considerable abundance as a cold water-insoluble free aglycone. The color of red varieties is owing to a cell sap-soluble anthocyanin which occurs also in the outer epidermal cells, and in red varieties obscures the yellow flavones which are also present. Associated with the flavones, colorless water-soluble phenols are always found in the dry outer scales, from which protocatechuic acid and catechol have been isolated [Link *et al.* (28, 29)]. These are toxic to spores of the smudge fungus and, being water-soluble and present in considerable quantity, they diffuse into the infection drop on the surface of the host and there prevent germination and penetration. This is a case, therefore, which is unusual in that it prevents penetration and the resistant character is expressed in the dead tissue at the portal of entry, and depends upon the fact that the chemicals involved are water-soluble and diffuse to the surface.

The importance of the situation just stated in effecting resistance is shown by the fact that if the colored fleshy scales are exposed to the pathogen without the protection of the outer scales they are invaded and parasitized as promptly and effectively as scales of white bulbs. This sometimes occurs in nature when outer colored scales are split, exposing part of the fleshy scale to the organisms in the soil. It can be demonstrated readily by removing the dry colored scales and exposing the fleshy scale to the pathogen in a moist chamber. Here the fungus penetrates equally well in colored and in white scales, although in the former the colored flavones are present in the epidermal cell. The difference from the usual case in nature is extremely important, however. In nature the toxic materials of the dry outer colored scales are free to diffuse to the surface and act as fungicides; in the living epidermal cell of the fleshy (colored) scale they probably do not exist as free phenols but are combined with other substances and, being confined by the plasma membrane, they do not diffuse to the surface. The fungus then penetrates the cuticle unimpeded and begins digestion of the subcuticular wall. As this process gets under way it can be observed that one of the first signs of breakdown in the epidermal cell contents is the disappearance of color compounds. Presumably phenols if they exist in the free state in such cells are destroyed or polymerized and are no longer inhibitive to the fungus which is about to enter the cell lumen.

The above picture is given in some detail because it illustrates what may often be the case in disease resistance. In this instance definite inhibitive substances or their precursors are apparently present in the outer living cell but are functionless as the resistant principle because either they are not present in a toxic form or the mechanisms of penetration and infection are such as to preclude their effectiveness on the invader. The fact that the outer scales of the onion bulbs cease to be living tissue and become desiccated enclosures of the fleshy living tissue is the basic reason why colored varieties are resistant since the toxic principles are then diffusible and functional.

When, perchance, the outer colored scales are split, permitting exposure of the fleshy tissue to inoculum in the soil, the disease develops as in a susceptible variety. In fact, some colored varieties when tested rigidly show little resistance because they have one or two thin outer scales at any one time and these slough freely so as to expose the fleshy scales [Hatfield *et al.* (21)].

Another case of resistance associated with phenolic substances has been reported upon recently by Johnson & Schaal (27, 39). Common scab of potato is incited by a soil organism, *Streptomyces scabies* (Thax.), Waksman and Henrici, which invades the periderm tissues of young tubers. After penetration the host responds by proliferation of periderm cells and the formation of characteristic scabby lesions follows. Johnson & Schaal (27) showed that the periderm cells of potato tubers contain chlorogenic acid and this is consistently higher in the periderm of varieties proved to be highly resistant. The close correlation of resistance and higher chlorogenic acid content suggests that the latter serves in some way to exclude or retard the pathogen at the threshold. Simultaneous but independent work by Cooper *et al.* (10) on the histology of potato scab has thrown new light on the host-parasite relation. In susceptible varieties the tubers in the early stage of their development show a distinct tendency to contain at the surface several layers of dead cells in which the pathogen becomes established as a saprophyte. In response to the presence of the organism the underlying living cells become meristematic and the proliferating layers proceed to develop and form the scab lesion. The proliferation appears to result from some materials which diffuse from the dead layers containing the pathogen; the pathogen stimulates division of the uninvaded living cells. In resistant varieties the living periderm is not covered with layers of dead cells or, if present, their number is very insignificant. Cooper and associates reason that the fungus is thereby not encouraged to become established at the surface and in consequence the proliferating stimulus is lacking. It may seem that the two theories of the nature of resistance are in conflict but further study may show that they are not entirely incompatible.

To return to the case of onion smudge, there occur within the fleshy scales of the host pungent materials which are given off by crushed scale tissue. These materials, which contain allyl sulfides, have long been known to have certain bactericidal and fungicidal value and are highly toxic to the smudge fungus [Walker *et al.* (55)]. It again becomes a paradox to find that *C. circinans* parasitizes onion rather than numerous other plants which it readily penetrates. What it is that determines that onion, containing two types of potential toxic inhibitors, should become the favorite host of this parasite is still a mystery. It disclaims the oft-repeated conclusion, however, that the mere presence of a material in the extract of host tissue which is toxic to a pathogen of that host, contributes to the resistance of the host. Several possibilities which favor the pathogen are always to be considered. The toxic material in a crude extract may not be present in the free fungicidal form in the living cell. It may be present in a nonlethal form, e.g., as a gly-

coside. It may be held in a portion of the cell contents not contacted by the pathogen. It may be destroyed in advance by excretions of the invading organism.

The phenolic substances and the volatile sulfides of onion are not specific to the smudge fungus. The phenolic substances, by the same mechanism as in the case of smudge, render colored bulbs resistant to *Diplodia natalensis* Pole-Evans [Ramsey *et al.* (36)] and to *Botrytis* spp. [Walker (49)]. The former invades the dry outer scale as does the smudge organism, and it is limited to white varieties generally as is smudge. The *Botrytis* fungi, however, normally enter through neck wounds, often with little or no contact with the dry outer tissue; in consequence, again, the mechanism of penetration is extremely important, and the resistant principle may be prevented from operating. Another common pathogen of onion bulbs is *Aspergillus niger* van Tiegh. This fungus is not affected by the phenol compounds of onion [Hatfield *et al.* (21)]. In fact, it is reported to produce protocatechuic acid as a metabolite when grown on certain media [Butkewitsch (6)]. Colored and white varieties are about equally susceptible to black mold incited by *A. niger*.

The importance of the volatile sulfides in restricting the advance of bulb invaders is not easy to evaluate by laboratory assay technique because one is never sure that he is in any sense simulating the actual conditions which prevail in nature. One way of approaching this is to compare mild and pungent varieties of yellow, red, and white classes under natural field conditions [Hatfield *et al.* (21); Owen *et al.* (33)]. This was done and inoculum of *C. circinans*, the smudge fungus, *B. allii*, a neck-rot fungus, and *A. niger*, the black mold fungus, were all provided. In one series of replicated plots natural climate prevailed; in a parallel series of plots the environment was made more favorable by frequent overhead irrigation. When the bulbs were rated and an index for each disease was calculated after a storage period, it was shown conclusively that pungent varieties were significantly less severely affected by smudge and by neck rot than the corresponding mild varieties in each color class. When the color classes were compared there was a wide difference between colored and white classes as to smudge and a significant but lesser difference as to neck rot. When the environment was made more favorable for the diseases the differences between the colored and white classes were less but still significant as to smudge but they were not significant as to neck rot. Under most favorable conditions for disease development the phenolic compounds were most influential in contributing to disease resistance in the case of smudge, while the volatile sulfides and probably other materials contributing to pungency were most influential with reference to neck rot. Neither factor had a consistent effect on resistance to black mold. Thus, in the case of the onion, the influence of volatile sulfides and associated materials in determining degrees of varietal resistance was demonstrated most conclusively by field studies rather than by laboratory assay.

Another case will now be discussed in which a potent antibiotic within the

host tissue was found to have no measurable influence on varietal resistance. It is well known that various parts of cruciferous plants contain mustard oils. The longest known of these is allyl isothiocyanate, which occurs in seeds, leaves and stems of black mustard [*Brassica nigra* (L.) Koch] and in some other cruciferous species as the glycoside, sinigrin. Upon crushing of the tissue the enzyme, myrosin, breaks down the glycoside and releases the volatile mustard oil. The mustard oil which occurs in black mustard roots and in roots of some other crucifers, presumably also as a glycoside, is β-phenylethylisothiocyanate [Stahmann *et al.* (45)]. Both of these isothiocyanates are much more toxic to spores and mycelium of several fungi tested than is allyl sulfide [Walker *et al.* (56); Pryor *et al.* (35); Hooker *et al.* (23, 24)]. The glycoside sinigrin, however, has no toxicity. Added to culture fluids in the pure form it has no effect on growth, but the addition of a slight amount of crushed mustard tissue provides sufficient enzyme to release the oil and the medium becomes highly toxic. It has been claimed that in the mustard plant the enzyme is contained in some cells and the sinigrin in others, and that the oil is not released until the tissue is macerated and the contents of the two types of cell are allowed to mix. It may be, however, that the enzyme and glycoside are contained in different parts of the same cell and that in the uninjured cell the enzyme is held inactive by inhibitors.

Rochlin (38) claimed in 1933 that the common observance that black mustard is highly resistant to the clubroot fungus, *Plasmodiophora brassicae* Wor., was owing to the fact that it contained a much higher amount of the toxic oil than susceptible species. Superficially, this appears to be a plausible explanation of the chemical nature of resistance. It is not unlike innumerable observations recorded in the literature in which the occurrence of one or another fungitoxic or bacteritoxic material is found in the resistant variety and, in lesser degrees, in the susceptible variety. The case of mustard oils in relation to resistance to clubroot has been studied extensively in our laboratory and the major results of this investigation will now be reviewed.

The clubroot organism penetrates the cruciferous rootlet from the soil as an amoeboid protoplast and migrates from cell to cell as it enlarges. It eventually gives rise to a large plasmodium which occupies most of the enlarged cell lumen and divides into innumerable uninucleate cells which become the spores of the organism. This obligate parasite is thus an intracellular one which develops in close association with the host protoplast and cell vacuole. Its effect upon invaded and uninvaded host cells as causal factor in hyperplasia and hypertrophy need not be discussed in further detail here.

The first step in our investigation was to determine whether or not all plants of black mustard were resistant. In an assemblage of seed collections from various parts of the world it was found that they were not [Walker (50)]. Some collections consisted of plants most of which were susceptible while in other collections most plants were resistant. By pure lining from these stocks a highly resistant and a highly susceptible line were secured and

used for further study. Both proved to be about equal in mustard oil content which was the first blow to the contention that mustard oil was the resistant principle; moreover there was no difference in myrosin activity to be found in the two lines [Stahmann *et al.* (45)]. When the resistant line was grown in quartz sand culture and watered with a balanced nutrient free from sulfur the plants showed no detectable mustard oil [Pryor (34)]. However, they were equal in resistance to those which received a normal supply of sulfur and contained the usual amount of sulfur oil. No evidence was obtained to indicate that the clubroot fungus or any of several others tested produced the enzyme which hydrolized sinigrin. Therefore, it might be postulated that the plasmodium of the clubroot organism could develop and grow in the host cell in the presence of sinigrin without necessarily becoming exposed to the toxic sulfur oil. This question has not been answered but the fact remains that there is no demonstrable relation between the toxic principle and the resistant principle. Since this work has been completed, considerable success has been achieved in developing club-root-resistant lines within the kohl group (*B. oleracea* L.) which do not contain allyl isothiocyanate and only a relatively small amount of the β-phenylethylisothiocyanate [Walker & Larson (53)].

In a further study of resistant and susceptible mustard tissue other possible keys to resistance were sought. This investigation turned toward possible differences in acidic and phenolic compounds [Smith *et al.* (43)]. Turnip (*B. rapa* L.) varieties, Purple Top Milan, Extra Early White Milan, and Snowball were shown to be highly resistant to the Wisconsin collection of the pathogen, while Shogoin was uniformly susceptible and Cowhorn contained 25 to 40 per cent susceptible individuals [Walker (51)]. Cabbage (*B. oleracea capitata* L.) was used as representative of very susceptible crucifers. A highly resistant strain of rape kale (*B. napus* L.) and a resistant and a susceptible strain of black mustard were included in the investigation.

When extraction and fractionation methods were applied it was found that the ether-soluble, strong acid fraction showed the most consistent differences in toxicity between extracts from susceptible and resistant turnips. Acid extraction or hydrolysis tended to increase toxicity levels of extracts of both types of tissue and, under certain conditions, seemed to increase differences between them. However, these experiments revealed that the titrable acidity of acid fractions varied considerably in different tissue samples and caused marked variation in the pH of extracts used in testing toxicity. A study of the influence of pH on the toxicity of turnip extracts [Smith *et al.* (44)] showed that the greater part of the toxicity of unbuffered extracts was probably due to this increased fungicidal activity of strong acids which cause low pH values. It seemed probable that the first results obtained in which differences in toxicity of susceptible and resistant tissue extracts were obtained would not have appeared if the pH effect of acids on toxicity had been eliminated.

Application of Newton & Anderson's (31) method of extraction of phenols

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to fresh turnip tissues showed a possible correlation of sap-soluble phenol with resistance. It was found, however, that the major part of the extractable phenols was freed only by acid hydrolysis or autolysis. The latter methods still produced no clean-cut correlation of phenol content and resistance, and the relatively low concentrations might argue against any relation to disease resistance. It is possible that small differences in pH of resistant and susceptible tissue may cause relatively large differences in the fungicidal effectiveness of certain cellular constituents. Although the present evidence fails to demonstrate that resistance to clubroot is due in any appreciable degree to readily extractable phenolic or acidic toxic constituents of the root tissue, the possibility remains that fungicidal compounds are produced during invasion by the pathogen in response to special stimuli or are caused by derangement of normal metabolism. The methods employed would hardly have detected mechanisms of this kind.

Greathouse & Watkins (19) pointed out that *Mahonia trifoliata* Fedde and *M. Swaseyi* Fedde which are highly resistant to the cotton root-rot fungus [*Phymatotrichum omnivorum* (Shear) Duggar], contain in the roots the alkaloid, berberine, in concentrations many times greater than that required to prevent growth of the fungus *in vitro*. The fact that the alkaloid is contained in a continuous zone of parenchyma cells just beneath the periderm is considered by them to indicate that it is an important factor in determining resistance. A similar association was found by Greathouse (17) between the alkaloids sanguinarine, chelerythrine, and protopine present in the roots and rhizomes of *Sanguinaria canadensis* and resistance of that species. Greathouse & Rigler (18) also associated alkaloids with the high resistance of a number of species of the Amaryllidaceae.

The complex nature of disease resistance becomes even more obvious when we consider diseases incited by pathogens which have numerous physiologic races. This situation prevails in the groups of fungi members of which are obligate parasites, e.g., rusts, powdery mildews, downy mildews. The distinction between races is based upon the faculty of a given race to parasitize successfully a certain variety or varieties of the host and not certain others. The work with flax rust has shown clearly that these highly selective properties of the races are gene-controlled [Flor (12, 13, 14)]. A number of distinct genes in the fungus each control a parasitic property which is effective upon only certain host varieties, while within the host varieties there is another series of genes each of which controls independently resistance or susceptibility to one or more of the distinct gene-controlled parasitic properties of the fungus. Herein lies the fundamental basis for physiologic races. It is likely that the same general situation prevails in many rust diseases. Resistance is not expressed at the portal of entry but after invasion and in the process of intracellular establishment and development of haustoria in the host cell. While we know nothing about the chemical nature of rust resistance it is obvious that no simple toxic material in the resistant host cell such as the phenols suggested by Newton *et al.* (31, 32) could explain it. The picture

suggests that, in such fungi as the rusts which will not grow on any ordinary culture medium, the availability of highly elaborated growth factors such as vitamins, polypeptides, and proteins is essential and, further, the presence of very exacting combinations of enzymes is necessary for fungus growth. Resistant cells may lack only one such component. Physiologic races may differ only in requirement of one component. The complexity of this situation has so far baffled any attempt at biochemical elucidation. Recently electrophoretic and chromatographic analyses of protein extracts of resistant and susceptible wheat seedlings have been used by Barrett & McLaughlin (2) in an approach to this problem.

There are other cases of pathogens which have numerous physiologic forms or races which are not so obligatory in their parasitism, and which, therefore, lend themselves a little more readily to biochemical approach. One such group is the vascular fusaria. The species *Fusarium oxysporum*, Schlect., contains numerous physiologic forms each of which attacks a single or a group of closely related host species. Thus, we have form *vasinfectum* on cotton, form *lycopersici* on tomato, form *conglutinans* on crucifers, form *niveum* on cucurbits, form *lini* on flax, form *pisi* on pea, form *cubense* on banana, form *tracheiphilum* on cowpea, etc. These forms have no constant morphological differences and are distinguished from one another by their property of becoming established in and inducing chlorosis, vascular browning, and often wilt of the particular hosts to which they are specialized. There do exist races within some of the forms which differ in their effect upon varieties within the host species, or upon closely related host species. Throughout this group of hosts and pathogens, there occur, as in the rust disease, individuals, lines, or varieties within a host species which are resistant to the form which is pathogenic on that species. In some instances, the resistance is of a high order in that it permits penetration but prevents establishment of the fungus in the root system of the resistant host. Such cases of high resistance to a given form or race within a form, as shown in the cases of cabbage, pea, and tomato, are controlled by single-gene pairs in the host. The fungi grow readily on common laboratory media. They invade their host plants through young root cortex, cross the latter with relatively little injury and then develop in xylem elements of the fibrovascular bundles of root and stem to which tissue they remain confined during most of their pathogenic activity. In this group of pathogens, therefore, we have organisms which resemble rusts in their high degree of specialization but which are also saprophytic enough that some of their metabolism can be studied in culture media.

The work in this laboratory has been concerned first with the tomato form and has extended to forms pathogenic on pea, cabbage, and cotton. The first approach has been to study the nature of host-parasite interaction in the susceptible host. Much past literature has accumulated in attempts to prove that toxic materials produced as metabolites in pure culture explained the action of the fungus *in vivo* on the host. Other investigators have maintained that plugging of the xylem elements is responsible for wilt. Our

studies have shown that in culture media the tomato wilt organism produces pectolytic enzymes, largely pectin methyl esterase (PME)[2] and a relative small amount of polygalacturonase (PG)[2] [Scheffer & Walker (41); Scheffer & Gothosakar (40); Gothoskar *et al.* (16)]. More recent work has revealed that since the latter enzyme produces little or no monogalacturonic acid it may be more properly classed as a depolymerase (DP).[2] This finding supports the theory that the pectolytic enzymes of fusaria break down pectin to produce large soluble fragments which accumulate in the vascular stream and eventually cause plugging. The plugs can be readily demonstrated as consisting of a pectin gel by their staining properties with ruthenium red. DP activity is essential for formation of vascular plugs inasmuch as PME alone does not produce wilting. Therefore it appears that the DP secreted by the fungus acts upon the insoluble pectin of the vascular elements to convert it to a partially degraded soluble form of pectin which passes into the vascular stream and accumulates there until it eventually plugs the conducting vessels. A similar enzyme has also been reported to be produced by *Pythium debaryanum* [Gupta, (20)], *Byssochlamys fulva* [Beaven & Brown (3)], and *Erwinia aroideae* [Wood (59)].

The first step in disease development is plugging of the major water and mineral channels of upward flow. Transpirational studies show a downward trend in water loss beginning as soon as or slightly before wilt symptoms appear. This accounts for only one phase of the disease syndrome. Another common early symptom is epinasty of lower leaves. This may well be due to the unusual accumulation of ethylene possibly produced in the leaves which is impeded in the normal downward flow at the point of the plugging. Ethylene may also be produced by the action of enzymes which attack pectin [Dimond & Waggoner (11)]. Another important phase of the disease syndrome is vascular browning, which consists largely of pigment produced in the xylem elements and absorbed by the secondary cell walls. Since the type of pigment suggested melanins it was postulated that phenolic compounds were involved. This hypothesis is strengthened by the observation that dilute solutions of several phenols when allowed to move up the xylem of tomato cuttings with the transpiration stream produced the typical browning of the vascular wall. This showed that sufficient polyphenol oxidases were present in the xylem cells to polymerize the phenols to melanins. It thus appears that the action of the fungus enzyme on the pectin in the cell wall results in a release of free phenols into the sap stream.

With the above elucidation of the nature of disease development the next step was to determine how specific was the action of the enzyme metabolites (degraded pectin) produced by different forms of *F. oxysporum* toward their respective host plants. One might easily be led to the assumption that herein lay the secret of fungus specialization and of host resistance. Such proved not to be the case. When enzyme preparations were made from the tomato form and applied to the vascular system through cuttings, plugging, browning, and wilting occurred in resistant and in susceptible tomato, in cotton,

in cabbage, and in pea. Likewise, preparations from the cabbage form, the pea form, and the cotton form acted effectively on all the plant species tested [Winstead & Walker (57)]. It is thus clear that the major disease-inducing principle, which resides in pectolytic enzyme production, is common to all the forms and the selectivity of forms for specific host plants and resistance of varieties within species toward a given form has apparently nothing to do with the disease-inducing principle.

We have in tomato a high degree of resistance controlled by a single dominant gene. The fact that such a wide difference is controlled exclusively by a single gene would seem to suggest that the basic difference is one involving a single, or relatively few, enzyme systems and the products of cell metabolism which they control. The location of the resistant principle has been claimed by some to reside in the root system of the tomato [May (30); Heinze & Andrus (22)]. This assumption might follow from the fact that the pathogen penetrates only the cortex of resistant roots and does not become established in the xylem although no morphological barriers can be discerned. That the resistant principle is well distributed throughout the plant has been shown recently by Scheffer & Walker (42). By an unusual handling of inoculation it is possible to distribute microconidia throughout the vascular system of resistant or susceptible plant within a few hours. This was done by inserting stem cuttings of resistant and susceptible tomato plants in a suspension of spores under conditions favorable for transpiration. After a few hours when the fungus could be isolated readily from throughout the leaf and stem vascular system, the cuttings were removed to a mineral nutrient solution until adventitious roots were produced when they were transplanted to sand watered with the same solution. The temperature was then raised to about 27°C. which is the optimum for disease development. Within the usual 10-day period wilting of leaflets appeared in both resistant and susceptible plants. In the susceptible plants the typical syndrome of disease ensued including chlorosis, vascular discoloration, epinasty, and eventually permanent wilt. In the resistant plants, however, after about two days of wilting of the leaflets the plants recovered and continued to grow in a normal manner although the fungus could be isolated from the petioles of leaflets for a period of ten days and from the lower stem for as long as 35 days. When such plants were examined histologically the fungus was located in primary xylem elements but in localized areas. Moreover, it had failed to move out of these infected locations as it did in the susceptible plants, and secondary xylem gradually surrounded the infected primary xylem. One might easily be led to misinterpret this histological picture as one of mechanical blocking off of the pathogen by the resistant host. On the contrary, we are inclined to interpret it as one in which the fungus was unable to multiply and excrete PME and DP sufficiently to become an effective pathogen in the resistant host while the "walling off" of the infected pocket was a normal growth procedure of the otherwise normal host tissue.

What it is that makes the resistant plant resistant is a mystery. It is a character which is not altered or broken down by changes in temperature or host nutrition although these factors do affect, decidedly, the extent of disease development in susceptible or intermediately resistant plants [Walker & Foster (52); Foster & Walker (15)]. It is possible, however, to convert the highly resistant into a susceptible plant. If resistant cuttings, which are inoculated through the vascular system with spores, are inserted in solutions of 2,4-dinitrophenol, thiourea, sodium diethylcarbamate or sodium fluoride at concentrations which are not perceptibly injurious to uninoculated plants, resistance breaks down and the cuttings go through the typical disease syndrome. So far, the only substances found to break down resistance are those which inhibit the activity of respiratory enzymes. It is also important to note that it has been shown that as a susceptible tomato plant becomes diseased respiratory activity declines. This can be shown by determination made by excised leaflets [Bloom (5)] or with sections of stems in the Warburg apparatus. Presumably, as the fungus functions as a pathogen it brings about an interference with the respiratory enzyme systems in the susceptible plant. These results suggest that in the resistant plant there is continuously being formed some substance which, when it accumulates to a certain level, becomes toxic to the fungus so that it blocks its advance. The formation of this substance seems to require energy from phosphorylation reactions for when these are blocked by 2,4-dinitrophenol resistance is quickly lost. This substance must also be either very labile or is being continuously metabolized to nontoxic materials for extracts of resistant plants are not toxic to the fungus. The effect of other inhibitors which break down resistance could also be explained by their action on energy-releasing reactions associated with respiratory processes. The growth of the fungus thus may actually increase the susceptibility of the plant to the disease as it also decreases plant respiratory activity.

The basis of the specific pathogenicity of each form for a certain host group has still not been determined. Does the key reside in a specific attraction of the host group for its form of the pathogen and none other? Or is the basis that of a particular metabolite of a given form which breaks down resistance only to that form and none other? These questions are at the center of the problem which, once unraveled, will probably throw important light on the cases of rusts, smuts, and mildews. A significant lead toward this problem has been developed by Winstead & Walker (58) who studied the heated filtrate of culture of the cabbage and cotton forms of *F. oxysporum* in which the nonspecific disease-inducing enzymes were inactivated. When plants were fed the heated filtrate at certain dilutions the response of susceptible cabbage to the filtrate of the cabbage form and of susceptible cotton to that of the cotton form was to show vein clearing and chlorosis which are suggestive of the early signs of the disease. Vascular browning and wilting did not occur as was to be expected since the pectolytic enzymes had been removed. The effect of the

heat-stable metabolites was noticeably more specific to the susceptible host variety than to other varieties or species. If this metabolite can be isolated from other material in the filtrate it may substantiate the hypothesis that each form of *F. oxysporum* produces a metabolite which diffuses slightly in advance of the fungus and in so doing predisposes the susceptible variety of the host species to this particular form and to it only. Once established, by the aid of this specific metabolite the form may thus proceed to induce the typical disease syndrome.

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XUM

ADAPTATION OF PLANT PATHOGEN TO HOST[1]

By J. J. Christensen and J. E. DeVay[2]

Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota, St. Paul, Minnesota

To avoid ambiguity, the term adaptation, as used here, means an acquired ability, a protoplasmic change, a temporary change, one not involving an alteration in gene complement. This implies that an organism may acquire and transmit the ability to do something that it either could not do originally or could not do as well, although no genic change was involved. The term adaptation also implies reversible changes when the stimulus is removed and therefore places an additional burden on the experimenter if he wishes to justify its use.

The nature and importance of genetic changes and phenotypic variation and variability in microorganisms is not only of great scientific and academic interest, but is also of considerable practical importance in connection with taxonomy, biochemical syntheses, industrial mycology, epidemiology, breeding for disease resistance, and other control practices. The growing interest in this field of work is indicated by the large number of recent publications on variability and genetics of microorganisms. Stakman & Christensen (137) have recently summarized the range and nature of variability in plant pathogens, while Christensen & Daly (29) have summarized the information on adaptation in fungi.

New varieties of crop plants have been produced repeatedly, only to succumb in a relatively short time to "new " races of destructive pathogens. How did these races or biotypes evolve? In many cases the nature of their origin is unknown. Some believe they arise by mutation, hybridization, heterocaryosis, or nuclear dissociation, whereas others believe that new races arise by adaptation. However, recent studies by numerous investigators strongly indicate that nuclear changes in many species of fungi are common; therefore, many of the changes hitherto attributed to adaptation are probably the result of genetic changes (70, 85, 115, 137).

Pathogenic fungi differ greatly from each other in their nuclear makeup; some are uninucleate while others are multinucleate; some are haploid, others are diploid; and yet others may be dicaryotic or heterocaryotic. Some pass through two or more distinct nuclear phases and oftentimes the pathogenicity of the organism is directly related to the nuclear phase involved (85, 126, 137).

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Knowledge of nuclear phenomena is pertinent to the understanding of variations in any organism. In the case of herterocaryons, one nuclear type may increase at a greater rate than the others and consequently this may lead to a gradual phenotypic change. In fact, Beadle & Coonradt (6) demonstrated that the proportion of genotypes in a heterocaryon may bring about differences in its rate of growth. Some investigators believe that pathogens growing on various substrates or in otherwise different environmental conditions lose the ability to produce certain enzymes, or become deficient for one or more growth-controlling substances, and that this may account for an apparent loss of virulence. These workers also believe that the loss of virulence can be restored simply by passing the organism through the host or by growing it on suitable substrates. They contend that these changes are temporary modifications and are not permanent or genetic changes. However, others believe that the pathogen is unstable, and that genetic changes account for its loss or gain in virulence. It has been shown that biotypes of some races may survive equally well in mixtures on certain media and hosts while not on others. Hence, selective media and certain hosts may serve as a means of selecting biotypes which may be already present in the original inoculum or may arise as a result of mutation.

There is good evidence that the virulence of certain pathogenic organisms may be increased or attenuated by growing them on different media (11, 19, 25, 29, 31, 98, 165, 166). Age of culture and the temperature at which the inoculum is grown also may influence the initial parasitic ability of a pathogen. The frequency, extent, and range of these modifications or changes in fungi, induced by substrates in which they were grown, have never been thoroughly investigated.

Although various reasons have been given for the loss or gain of virulence of certain pathogens when grown on artificial media, the true nature and cause is often unknown. However, mutations are common in most fungi and, as a result, inoculum produced on nutrient media often consists of two or more biotypes of different virulence even though derived from single spores (28, 31, 126). This fact is often overlooked in studies involving "adaptation" and hence, erroneous conclusions are often reached.

Changes in environmental conditions often affect the associations between plant pathogens and their hosts. Whether these changes influence the virulence of the pathogen, the susceptibility of the host, or both, is usually difficult to determine. However, in some instances where an infected plant is subjected to small environmental changes and a marked increase in disease results, an adaptive change in virulence of the parasite may be responsible. Such changes also may occur in plant pathogens whose hosts apparently become more susceptible to certain diseases with age. Although it is not always easy to distinguish between phenotypic variability and genetic variation, it is of course extremely important in studies on adaptation, to recognize the range of variability in disease expression.

XUM

Variability of Disease Expression in Relation to Environment

As plants age and are exposed to various environments, the free metabolites of their cells fluctuate in concentration (76, 83, 144) and probably in kind. The influence of various balances of free metabolites in plant cells on the virulence of invading parasites, until recently, has received limited attention (91). Yet, the effect of variations in the composition of the host may well stimulate or activate adaptive mechanisms in the parasite which are expressed in disease development.

Temperature.—The influence of temperature on disease expression in plants has been recognized and studied for many years. Extensive and valuable contributions toward the clarification of this phenomenon have been made by Jones *et al.* (84) and Dickson (45) who pioneered in this phase of pathology by concentrating their attention on the relation of soil moisture and temperature to the development of plant diseases. An excellent review by Wingard (167) has summarized the earlier studies on the relation of temperature to disease development.

Recent discoveries concerning the sensitivity of rust reactions on cereals to fluctuations in temperature have emphasized the importance of knowing the physiological potentialities of the rust fungi, particularly in respect to adaptive mechanisms. Extensive testing of rust races at the Minnesota Agricultural Experiment Station cooperating with the Federal Rust Laboratory has demonstrated the necessity of controlled temperatures when identifying rust collections on differential varieties of *Triticum* spp. (102, 136).

Many investigators have reported marked changes in rust infection types on cereals following exposure of host plants to slightly higher temperatures (1, 13, 40, 60, 66, 69, 81, 102, 113, 134, 136). Peturson (113), Hingorani (69), and Simons (134) observed that certain physiologic forms of *Puccinia coronata avenae* on certain moderately resistant oat varieties could be induced by a temperature increase of approximately 10 to 20°F. to produce pustules or infection types of greater magnitude. Similar findings were made by Ali (1), Bromfield (13), Darley & Hart (40), Grimm & Dickson (60), Hart & Stakman (66), Johnson & Newton (81), Mohamed (102), and Stakman (136), in the pathogenicity studies of *P. graminis tritici* on *Triticum* spp.

The effect of temperature changes on the development of other plant diseases has been reported by several investigators (56, 58, 112, 114). Pound (114), in greenhouse studies of early blight of tomato (*Alternaria solani*), found that disease development caused by four different pathogenic isolates was much greater at 16°C. than at 28°C. with a gradient of disease severity decreasing from 16° to 28°C. Similar temperature effects were found by Paulus & Pound (112) who reported that with dilute inoculum of the gray leaf spot fungus, *Stemphylium solani*, disease expression was very slight at 28°C. and moderate to severe at 16°C. With concentrated inoculum, the disease was equally severe at 16°, 20°, and 24°C. but considerably reduced at 28°C. The response to powdery mildew (*Erysiphe graminis tritici*) of 15

spring wheat varieties grown in the greenhouse at 16°, 20°, 24°, and 28°C. was studied by Futrell (56). He found that the mildew reaction of Hope wheat was more sensitive to temperature change than that of the other varieties. Leaf blades of Hope were moderately susceptible at 24°C. but resistant at 16° and 20°C. Gerdemann (58), in pathogenicity studies of *Leptodiscus terrestris* on red clover, found that the varieties Midland, Kenland, and Dollard were completely susceptible to damping-off when seeded in warm (20° to 25°C.) infested soil. However, when seeded in cold (10° to 15°C.) infested soil, these varieties were more resistant. Older plants, too old to damp-off were killed by root rot when the soil temperature was raised to 20° to 25°C.

Plant nutrition.—The influence of host nutrition in relation to the development of plant diseases has been the object of a number of recent studies (3, 9, 16, 39, 49, 57, 83, 97, 144, 155 to 159, 162, 163). Older literature pertaining to this subject has been extensively reviewed and summarized by Wingard (167). In a series of greenhouse studies in which plants were grown in quartz or white silica sand and irrigated with a basal Hoagland solution whose concentration was varied by altering the total salt concentration or by changing the concentration of N, P, and K, Walker and co-workers found that disease expression in several host-parasite relationships was increased. Alterations in disease expression were found in black rot of cabbage (*Xanthomonas campestris*) and ring rot of tomato [*Corynebacterium sepedonicum* (155)], bacterial canker of tomato [*E. michiganense* (156)], tomato wilt [*Fusarium oxysporum* f. *lycopersici* (157)], cabbage yellows [*F. oxysporum* f. *conglutinans* (158)], cabbage clubroot [*Plasmodiophora brassicæ* (159)], cucurbit wilts caused by *Erwinia trachephila* in cucumber and *F. oxysporum* f. *niveum* in watermelon plants (162, 163), and in bacterial wilt of tomato [*Pseudomonas solanacearum* (57)]. Bloom (9) reported that high concentrations of potassium (KCl) on the foliage of tomato plants increased their susceptibility to wilt (*F. oxysporum lycopersici*), while high potassium concentrations supplied through the roots decreased disease. In studies on the infection of pineapple roots of *Phytophthora cinnamomi*, Anderson (3) found that a decrease of potassium from 381 p. p. m. to 146 p. p. m. in the nutrient salt solution of 1000 p. p. m. total salt concentration markedly increased the amount of root rot. McClellan & Stuart (97) found *Fusarium* rot in gladiolus corms to be increased by a nutrient solution high in nitrogen and low in phosphorus supplied to the plants in subirrigated greenhouse nutriculture benches. In contrast, Jones & Darling (83) observed in controlled nutritional studies on Triumph potatoes that low levels of nitrogen and magnesium accentuated early blight symptoms caused by *A. solani*. Three different magnesium levels in nutrient solutions supplied to two inbred lines of corn by Taylor (144) altered the nutrient-element composition of both inbred lines. Associated with the increase of magnesium in the nutrient solutions was a highly significant increase of this element in the leaf tissue with a significant decrease in the sulfur, potassium, phosphorus, and nitrogen content. These changes in

the nutrient solutions also altered the susceptibility of the plants to leaf spots caused by *Helminthosporium maydis*. Disease susceptibility consistently increased from the low magnesium levels to the high. In sand culture studies, Doak (49) found that excess nitrogen increased the susceptibility of wheat varieties to leaf rust (*P. rubigo-vera tritici*) whereas Daly (39) observed no marked changes in stem rust (*P. graminis tritici*) on Thatcher, Mindum, or Marquis wheats supplied with high concentrations of nitrate nitrogen. However, Daly found that Thatcher, at temperatures between 65° and 75°F. was completely resistant to race 56 (*P. graminis tritici*) when supplied ammonium nitrogen but more susceptible when supplied equivalent amounts of nitrate nitrogen. Browning (16) reported a breakdown of crown and stem rust resistance in seedling leaves of differential oat varieties when detached leaves were supplied with two per cent glucose or sucrose solutions at about 70°F.

Other Environmental Factors.—Several instances have been reported where increased disease susceptibility is associated with aging of host plants. Campos *et al.* (22) found that in crosses involving the wheat variety, Mentana, many lines which are resistant in the seedling stage to stem rust (*P. graminis tritici*) are susceptible in the adult stage. Similar observations on Mentana wheat infected with race 15B of *P. graminis tritici* were made by Lowther (89). An increase in susceptibility of resistant potato plants, during senescence, to late blight (*P. infestans*) has been reported by Conover & Walter (37). Likewise, Chamberlain & McAlister (24) found that brown stem rot of soybeans, which is caused by *Cephalosporium gregatum*, progressed more rapidly in plants after the pod-filling stage.

The reaction of the soil is another factor which may affect disease susceptibility in plants. It is generally accepted that *Actinomyces scabies* is more virulent on potatoes in alkaline or weakly acidic soils. Horsfall and coworkers (76), in attempting to explain this phenomenon, made experiments to determine the influence of calcium. They found that the calcium content in a tuber was related to the amount of replaceable calcium in the soil which, in turn, is governed by the reaction of the soil in calcium dosage. An explanation was proposed that calcium seems to exercise a role, independent of the hydrogen-ion, in the etiology of scab and that the amount of calcium in the tuber is proportional to the susceptibility of the host or virulence of the pathogen. The club root disease in crucifers is also related to soil acidity. Chupp (34) found that a soil reaction greater than pH 7.2 to 7.4 would inhibit club root while a reaction less than pH 6 would cause greater disease development. Similar results were reported by Schaffnit & Meyer-Hermann (130).

A relationship between light intensity and disease susceptibility was demonstrated by Hingorani (69) who found that the oat variety, Marion, grown at 23° to 25°C., was resistant to stem rust (*P. graminis avenae*) at 250 foot candles but susceptible at 7000.

Changes in concentrations of free metabolites in a host plant which have been induced by alterations of environmental conditions or plant age,

alter the nutritive environment provided for infectious organisms which, in turn, may stimulate or inhibit adaptive enzyme systems influencing the virulence of the pathogens.

In studies involving disease susceptibility of plants and the virulence of various plant parasites, careful consideration should be given to variables in environmental conditions as well as to different stages of host development to avoid erroneous conclusions.

The above results show the necessity of caution in generalizing regarding variation in disease expression. Sometimes it was difficult to distinguish clearly the nature of variation because of the influence of environmental factors either on the host or pathogen, or both. Sometimes the difficulty was perhaps due to questionable genetic purity of the original material.

Influence of Artificial Substrates on the Virulence of Plant Pathogens

Several studies have been made on the pathogenicity and virulence of certain fungi as effected by nutrition on artificial substrates (11, 19, 166). Westerdijk (165) has summarized much of the earlier information in view of the preservation of parastitism of certain fungi in pure culture. The influence of salicylic aldehyde media on *Pythium* isolates was reported by Bouchereau (11) who found that after repeated transfers on these media, some of the isolates caused more root necrosis on corn than the original isolates or the aldehyde alone. White & McIntyre (166) compared the pathogenicity in the field of eight monoascospore isolates of *Ophiobolus graminis* from a single ascus. They grew each isolate on three kinds of media prepared from straw, oat-barley, and soil, and then used these cultures to infest two types of soil so that the effect of the medium on pathogenicity could be measured. Five of the isolates were most virulent when grown on the medium prepared from straw while two isolates were most virulent when grown on the oat-barley medium. One isolate was nonpathogenic.

Mycoparasitism by *Rhizoctonia solani* was studied by Butler (19) who found that this fungus was avirulent to *Mucor recurvus* if the hyphae used for inoculum were grown on water agar, autoclaved fresh peas, or wheat germ; however, if grown on rice, autoclaved yellow corn meal, oat grain, or polished rice grains, it was highly virulent. He also demonstrated that parasitism by *R. solani* on *M. recurvus* was decreased if both fungi were supplied a pentose sugar as a carbon source.

The significance of these studies in respect to adaptation is enhanced by the rapidity in which pathogenic differences were brought about in pure cultures as a result of nutritional differences in the culture media. Whenever repeated transfer generations are required to produce a certain change in microorganisms, it is improbable that adaptation has occurred. Most likely, the growth of one or more biotypes, arising by mutation or heterocaryosis coupled with nuclear dissociation, has been favored by a particular medium so that the original characteristics of culture are masked or even lost.

Problems thus arise in the maintenance of various levels of pathogencity, in the stability of biochemical mutants, and in various other characteristics used in genetic studies of microorganisms in pure culture. Moreover, a knowledge of the effects of various nutrient substrates on virulence or pathogenicity is of great importance in testing the disease resistance of new varieties of crop plants. Whether these changes in virulence of the pathogens have occurred as a result of adaptation or by changes in the genetic make-up of the organism is difficult to determine. However, the purity of the original cultures and the rapidity in which the changes in virulence occurred, suggests that an adaptive mechanism might be involved.

Mixtures of Biotypes in Culture

It is well established that in fungi, a species may consist of numerous dynamic biological entities or biotypes (72, 82, 86, 121, 135, 136, 149). Like higher plants, races and biotypes of given pathogens differ in their nutritional requirements, in that certain substances, whether they be in host plants or in artificial media, may stimulate one biotype while inhibiting another. Thus, the biotype most capable of utilizing the nutrients and tolerating possible inhibitors in a particular environment has a much better chance of survival, providing other factors favor its development (29, 31).

Biotypes within the species of *Ustilago zeae*, *H. sativum*, *F. moniliforme*, *Gibberella zeae*, and *Colletotrichum linicola* differ strikingly, not only in cultural characters, but also in their tolerance to toxic materials (28, 31, 137). Certain biotypes thrived on nutrient media containing high concentrations of mercuric chloride, sodium arsenite, ethyl mercury phosphate, or malichite green, whereas other biotypes of the same species grew poorly or not at all (28, 29, 31, 137). Thus, by applying this information, it is possible to purify mixed or contaminated cultures. This is analogous to the inoculation of plant hosts with a mixture of pathogenic biotypes possessing different parasitic abilities. Such tests were made by Christensen (28, 29) who obtained results comparable to those obtained on artificial media.

In order to simplify the test, cultures of two races of *H. sativum*, one black and virulent, the other an albino and weakly pathogenic, were mixed together in various proportions and then used as inoculum. There was a tendency, after several passages through the host, for the albino race to be eliminated irrespective of its concentration in the original inoculum. If the proportion of the albino used as inoculum was less than 50 per cent, it seldom was isolated after the first passage through the host. Also, the greater the proportion of the black race, the more severe was the infection. In only one test was the black race eliminated. The reason for this exception was not ascertained. In all cases only the two original races were reisolated.

It has been repeatedly demonstrated that a number of mixtures of biotypes of *F. moniliforme* may survive equally well on certain media (28). Moreover, such mixtures may appear pure in cultural types, yet they are a composite of two or more distinctly different cultural biotypes. Their presence,

however, can be readily detected by comparing colonies on dilution plates or by making single spore isolations from mixed cultures. Mutations involving pathogenicity also occurred in these biotypes without being reflected in cultural changes, so that pathogenicity tests were necessary for their detection.

The fact that mutants occur and can be carried as a mixture in culture for a long period of time without detection may help to explain many erratic and puzzling results obtained in connection with so-called adaptation.

Competition for Survival Among Races of Plant Pathogens

The ability of certain races of parasitic fungi in a mixture to become predominant over the others has been studied by several workers (77, 93, 124, 148). Rodenhiser & Holton (124) inoculated susceptible Ulka wheat with two races of *Tilletia foetida* in paired mixtures with nine races of *T. caries* and studied their survival ability at Aberdeen, Idaho, and at Bozeman, Montana. They concluded that the two species and races of the bunt fungi differed greatly in their respective ability to survive in a susceptible wheat. Races of *T. foetida*, in general, were more aggressive than races of *T. caries*. Races of *T. foetida* usually predominated after the first pasage through the host. This was not always the case, as one race of *T. caries* eliminated *T. foetida* completely from a mixture after the seventh passage through the host. In 7 out of 18 paired mixtures, grown at Aberdeen, *T. foetida* completely eliminated *T. caries*. There also was a tendency for certain races within a species to predominate.

Similar studies have been made on the survival ability of certain races of *P. infestans* (148), *P. graminis tritici* (93), and *P. rubigo-vera tritici* (77). Thurston & Eide (148) demonstrated by successive transfers on Cobbler potatoes, that the survival ability of the "Cherokee isolate" of the late blight fungus (*P. infestans*) in mixtures with the "Cobbler isolate" was lessened with no apparent loss or gain of virulence by either isolate. Loegering (93) found that if a mixture of races 17 and 56 of wheat stem rust (*P. graminis tritici*) were grown for seven successive uredial generations on Ceres, Fulcaster, and Little Club wheats, all of which appeared to be equally susceptible to the two races, race 17 became predominant by a wide margin on Fulcaster and Little Club, while on Ceres it predominated slightly. When a mixture of races 17 and 19 was grown for six successive uredial generations on Mindum durum, Fulcaster and Little Club wheats, all of which appeared equally susceptible to the two races, race 17 predominated after less than six generations on Fulcaster and Little Club, while on Mindum race 17 predominated only slightly after six generations.

Competition among physiologic races of the leaf rust of wheat (*P. rubigo-vera tritici*) was studied by Irish (77) who mixed equal amounts of four races 9, 15, 58, and 126, and grew them together on the uniformly susceptible host variety Cheyenne. He found that races 9 and 15 were the dominant competitors while race 58 was the weakest.

The ability of one race of a pathogen to predominate over another after several transfer generations on uniformly suceptible hosts, might be due to a difference in the rate of spore germination or invasiveness of parasitic hyphae. Still another contributing factor might be the antagonistic effect of one race on another. However, Rodenhiser & Holton (124), after studying some of these factors were unable to account for differences in survival of species and races on a congenial host. Yet, the ability of certain races of the late blight fungus, cereal rusts, or smuts, to be more aggressive or more adaptive than other races on host plants, is probably related to a complex of many small differences which individually are almost undetectable among the various races.

Herterocaryosis and Nuclear Dissociation

Heterocaryosis, the presence of two or more genetically different nuclei in a spore, vegetative cell, or in a coenocytic hypha apparently is one of the common phenomenon by which new races or biotypes of fungi arise. Heterocaryosis occurs independently of sex (63), and can result from the fusion of spores or hypha of different biotypes. Mutation within the spore or hypha of a monocaryon also may result in heterocaryosis. A heterocaryotic condition usually results in dissociation or regrouping of different kinds of nuclei.

Hansen & Smith (64) and Hansen (63) have emphasized that anastomoses frequently take place between hyphae and germ tubes of plant pathogens within or between species. This phenomenon may result in an interchange of cell contents including nuclei. Hansen (63) studied the isolates from 30 genera of Fungi Imperfecti and concluded that most of the isolates were heterocaryotic. Hansen & Smith (65) made a special study of *Botrytis cinerea*, and found that the hyphal cells and conidia were multinucleate and, hence, could easily harbor nuclei of different origin. They paired distinctly different monocaryotic lines and observed hyphal fusions between them. Heterocaryosis apparently resulted, as single-spore isolations from the resulting cultures gave rise to not only the original types, but to intermediate types which continued to dissociate into other variants, for at least five generations. Hansen & Smith (64) grew an isolate of *B. allii* and one of *B. ricini* together in mixed cultures, and then compared the progenies resulting from single spores from these cultures. The two original lines were recovered as well as several others with different cultural types. These results indicate that the origin of new lines may result from hyphal fusions.

Dissociation frequently occurs in many fungi on artificial substrates and it may be rather common in pathogens on living hosts, yet only a few definite cases have been clearly demonstrated. Christensen & Graham (32) found that the heterocaryotic fungus *H. gramineum*, may dissociate into many distinct genetic lines when passed through barley. From one monosporous isolate they obtained 10 cultural races after a single passage through barley. Except for occasional sectors, culture types of lines reisolated from the host remained constant for many transfer generations.

Nelson & Wilcoxson (108) mixed urediospores of races 38 and 56 (*P. graminis tritici*) and then inoculated Little Club wheat with the mixture. In the next uredial generation the rust was transferred to the 12 varieties of wheat used in identifying physiologic races of stem rust. A new race was detected because of its high virulence on Khapli wheat, a variety highly resistant to the parental races. The pathogenicity of this race was unstable as indicated by its gradual loss of virulence during 30 transfer generations on Khapli. Its loss of virulence was probably due to nuclear dissociations as two new races in addition to the parental types were isolated from single uredia during the repeated transfer generations on Khapli. Cytological studies revealed that the urediospores of the virulent heterocaryon were 2 to 4 nucleate whereas the four races recovered from the heterocaryons were binucleate, a condition characteristic of most urediospores. Apparently there was an increase in virulence due to new nuclear associations; however, the gradual decrease in virulence was probably associated with nuclear dissociations. This is an excellent example where a mixture of urediospores, consisting of two races, may cause severe infection on hitherto resistant varieties, which neither of the original races could attack.

Dicaryosis, a condition usually preceding the formation of diploid nuclei in certain fungi such as the rusts, in which each cell contains two nuclei of opposite sex in close association, should be distinguished from heterocaryosis. Dissociation of these nuclei in certain fungi on artificial substrates, is fairly common and it also has occurred on living hosts (14, 17, 44, 62). Because of recent and important discoveries of heterocaryosis in *P. graminis tritici*, this problem should be thoroughly investigated in other rust fungi in which the alternate host is unknown or of little importance. Thus, evidence is accumulating that heterocaryosis and nuclear dissociations are important ways by which "so-called" adaptation occurs in some fungi.

Effect of the Host on Sporulation

There is a general tendency for many species of fungi to lose their ability to sporulate freely when grown continuously on artificial media. Many workers consider such changes as temporary and believe that this character can readily be restored by passage through a host or by the addition of certain growth substances to the medium. Thus, it appears to be a physiologic change rather than a genetic one. Perhaps both are involved. Several workers have reported an increase in sporulation following passage of poorly sporulating or nonsporulating isolates of fungi through the host, whereas others have reported no such changes. Dastur (41) restored the spore-producing ability of a variant of *Glomerella* sp. by passage through the host. Likewise Coons & Larmer (38) found that *Cercospora beticola* reverted to the original type after passage through a host. Conversely, Caldis & Coons (21) were unable to increase the fruiting capacity of a weakly sporulating variant of *Cladosporium fulvum* by its passage through tomato plants. Similarly,

Chilton (25), Christensen (26), Keitt & Langford (88), and Ullstrup (150), found that sporulation of isolates from several pathogenic fungi was not changed by passage through their respective hosts.

However, Christensen (28) was unable to increase the sporulating capacity of several monosporous isolates of seven species of fungi by passing them through the host for one or more generations. Negative results were obtained by passing three isolates of *G. zeae*, six isolates of *F. moniliforme*, and two isolates of *Diploida zeae* through corn plants; eight isolates of *H. sativum* through wheat and barley; four isolates of *H. teres* through barley; two isolates of *H. oryzae* through rice; and three isolates of *F. lini* through *flax*. On more than one occasion, however, variants of *H. sativum* were obtained directly from the host which differed in sporulation ability from the original cultures. Some isolates had an increased ability to sporulate, while in a few cases, the ability had decreased. Moreover, original isolates also were recovered and usually predominated. Changes in ability to sporulate were always associated with an alteration of cultural characters. These variants were as stable as the original cultures and did not revert to the parental type when grown on different nutirient media. Apparently mutation had occurred on the host or perhaps the host was inoculated with a mixture of biotypes as the inoculum consisted of many spores and hyphal branches. In either case, the host simply acted as a selective medium. It is significant that weakly sporulating types have been obtained directly from a host plant inoculated with a single conidium of a highly sporulating line of *H. sativum*.

It is, of course, not uncommon for weakly sporulating lines of many species of fungi grown on a nutrient substrate to give rise to variants that fructify more abundantly than the original line. One might expect similar results to occur on a host.

Effect of the Host on Cultural Characters

It is well known that the character of many fungal cultures, even those of monosporous origin, are greatly altered when grown on artificial media. There may be striking changes in color, growth habit, zonation, sclerotial production, mycelial tufts, rate of growth, and production of toxic materials and enzymes. Some consider these alterations as genetic changes; others simply as a phenotypic modification, a temporary change, an adaptation to a saprophytic mode of growth. If the latter is true, then pathogens should revert to the original type after one or more passages through the host. Burkholder (18) and Coons & Larmer (38) noted that certain pathogens had a tendency to revert to the original type after a sojourn on the host. In recent years much evidence has accumulated which indicates that most cultural changes are genetic in nature and cannot be modified by passage through hosts, provided mixed biotypes are not involved.

Keitt & Langford (88) could not alter the cultural characteristics of three haploid lines of *Venturia inaequalis* by four successive passages through

leaves of McIntosh apples. Ullstrup (150) passed 15 isolates of *G. zeae* through corn seedlings and concluded that the sojourn on the host, which was about one month, had no perceptible effect on the cultural characters. Similar results have been obtained with isolates of this fungus at Minnesota (28), although in one case there was a distinct cultural change. This result was based on the reisolation of the fungus from the host tissue. No attempt was made to ascertain the purity of the population at the time reisolations were made.

Chilton (25) attempted to rejuvenate 10 subisolates of *C. distructivum* with diverse cultural characteristics derived from a single monosporous stock culture. No changes occurred after five successive cultural generations on sterilized host tissue. He also passed three of these subisolates through two successive generations on living clover seedlings without modifying their cultural characters. Chilton (25) concluded that variants replaced the original type and the changes were genetic. This is supported by the fact that mutations were common in culture and when he plated out 986 spores, derived directly from a clover plant inoculated with a single spore, he obtained two distinct cultural variants. Therefore, even if changes did occur from a sojourn on the clover, they might have been due to mutations. Similar results have been obtained by Christensen & Schneider (33) with *H. sativum*. Christensen & Davies (31) and others proved experimentally that fast growing variants commonly replace original isolates when grown on nutrient media. This fact is highly significant, because cultural changes often are associated with a change in pathogenicity (31, 87, 137).

There is considerable evidence that cultural characters of certain species of *H. sativum* are not readily altered by passage through a living host. Brown & Miller (15) concluded that passage of certain isolates of *H. sativum* through several oats varieties had no effect on their morphology and on the symptoms they produced on host plants. During the past 20 years the senior writer and his co-workers have passed many variants of *H. sativum*, one or more times, through either wheat or barley. Reisolation usually gave the same cultural type as that of the original ones kept continuously on nutrient agar. Occasionally two or more cultural types were isolated from the same inoculated plant. This is to be expected, especially when more than a single spore was used to inoculate the plant, as mutations are common in culture and not uncommon in the host.

Ten isolates of *Diplodia zeae* were passed through corn seedlings in four successive generations and then compared on nutrient media with original lines kept continuously in culture (28). There were no significant differences between these lines in their growth rate, ability to utilize certain nutrients, and other cultural characters, including color and pycnidial formation. Changes, although not apparent, had taken place, as some lines appeared more or less pathogenic than the original cultures. These results emphasize the importance of not drawing definite conclusions from a limited number of experiments pertaining to adaptation and genetic variation.

XUM

Changes in Virulence of Plant Pathogens on Their Hosts

Between 1900 and 1920 many investigators believed fungi could acquire the ability to attack an immune host or even an unrelated plant species provided the host was injected with the appropriate substance (20). A few even believed that a saprophytic fungus could gradually be "educated" to become an active parasite on a given host (95, 96). These results have never been verified by modern techniques and are not generally accepted.

Rusts and mildews.—About that time it also was claimed that a parasitic fungus could acquire the ability to attack an immune host if the organism was cultivated for a number of generations on an injured host and then transferred to an uninjured plant of the same variety. By this means, Salmon (127, 128) in 1904, reported that powdery mildew of barley, *E. graminis hordei*, could be induced to grow normally on wheat. In 1925, Hammarlund (61) repeated Salmon's work and although he grew powdery mildew of wheat for 128 asexual generations on wounded barley leaves, the mildew never acquired the ability to attack uninjured barley plants. The fungus did not lose its virulence for the original wheat host after a sojourn of 125 generations on barley. Recently, Stakman and co-workers injured many barley plants by diverse methods, but were unable to induce *E. graminis* to attack hitherto immune plants (135, 137).

In the early part of the 20th century many workers believed rust and mildew fungi could readily adapt themselves to resistant or immune varieties. Magnus (94) suggested that new races of stem rust arose through the association with the host plant. Dietel (46) had similar views. In 1903 Ward (160, 161) made many experiments with leaf rust (*P. dispersa*) of brome grass, and concluded that the virulence of brome rust could be changed easily through its association with an appropriate host for one or more successive generations. He also concluded that there were "bridging species" of *Bromus* that enabled the rust to attack very resistant or immune varieties.

Thus, if the race of brome rust could not be transferred directly from one species of brome to another, the transfer could be made by passing the rust through a third species of brome, the "bridging species" which usually was taxonomically intermediate between the immune or highly resistant and the susceptible host. Freeman (53) obtained similar results with brome rust. Ward made no carefully controlled experiments to prove his assumptions concerning the existence of bridging species. The evidence presented by Ward and Freeman is too meager to draw definite conclusions.

Later Freeman & Johnson (54) concluded that the rust on wheat, *P. graminis tritici*, could be induced to attack oats after it had been increased on barley. They grew a collection of stem rust of wheat for several generations on barley and then transferred it from barley to wheat, rye and oats. Two of 54 oats plants inoculated, produced uredia and 10 developed flecks, whereas in the original inoculation of wheat no infection was obtained on 66 plants. There also was a tendency for a higher percentage of infection to occur on rye. According to Stakman & Piemeisel (140), if sufficient inocula-

tions of stem rust are made on wheat, occasionally infections will occur on oats by direct inoculation from the wheat. Since then it has been shown many times that infectivity of stem rust cannot be increased by successive passages of the rust through a particular cereal host. Johnson (80) reported that he could widen the host range of the rust on timothy (*P. graminis phleipratensis*) by using oats as a bridging species.

In 1911 Evans (52) crossed suceptible × resistant varieties of wheat (Woeloren × Bobs Rust Proof) to stem rust and found that rust from hybrids could attack both susceptible and resistant parents. He concluded that the hybrids could serve as a bridging species and thus enabled the rust to attack the resistant parent. He does not give a detailed account of the inoculation experiment, but his results can probably be explained by the introduction of new races of rust. Workers at this time were not aware that physiologic specialization occurred within the rust variety *P. graminis tritici* and hence did not realize the significance of using the same culture of rust throughout their entire experiment.

In 1910, Arthur (4) concluded that specialization of *P. graminis* broke down following the sexual stage on the barberry. Therefore the barberry acted as a bridging host between varieties of *P. graminis*. In 1926, Dietz (47) concluded that the buckthorn altered the infective capacity of different physiologic varieties of *P. coronata*. Today, these results can readily be explained on the basis of hybridization between biotypes belonging to different varieties of rusts. It was not until 1927 that Craigie (35, 36) proved that hybridization between races occurred on aecial hosts.

Salmon (127, 128) made many experiments with *E. graminis* in which he claimed that the resistance of the host was broken down. Thus, the mildew on *B. commutatus* would not infect *B. mollis* directly, but when passsed through *B. hordeaceus* it acquired the ability to attack *B. mollis*. There are no indications that Salmon gave adequate consideration to the possibility of contamination, which can readily occur with a fungus like *E. graminis*, whose spores are easily dispersed by air currents. This is particularly true when conclusions are based on infections of one or two leaves. In addition, the possibility of physiologic specialization was not given due consideration. Similar changes in parasitism in *P. infestans* as reported by Reddick & Mills (118) and De Bruyn (42, 43) is somewhat analogous to the bridging reported in the rusts and mildews.

It is only fair to point out that much of the work on bridging was done before biologists were aware of the fact that physiologic specialization occurred in fungi and that most races of fungi consisted of indefinite numbers of biotypes (51, 72, 87, 136, 137). Moreover, they were not aware of the fact that mutations, hybridizations, heterocaryosis and nuclear dissociations were common phenomena in plant pathogens. In most cases the source and purity of the inoculum was not indicated, and in many cases it may have been a mixture of biotypes, at least it is very doubtful if single-spore techniques were practiced. The cause of these pronounced changes is unknown

as no one has been able to repeat these early results with the more refined methods. Stakman & Jensen (138), and Stakman & Piemeisel (140) found no indication of adaptation, although the pathogen was associated with so-called bridging species, sometimes for many generations. Stakman, Piemeisel & Levine (141) maintained a race of *P. graminis tritici* for three successive years on barley; however, no new parasitic capabilities arose from this association. Stakman, Levine & Cotter (139) reported that a race of *P. graminis tritici* remained constant for 13 years at which time a mutant occurred which differed strikingly in pathogenicity from its parent.

Present studies (82, 109) indicate that mutation in rusts are not uncommon and must be considered as a possible cause of what sometimes seems to be adaptation. The recent work by Nelson & Wilcoxson (108) emphasized the need for much more information on the role of heterocaryosis and nuclear dissociation in the production of new biotypes.

Smuts.—Evidence continues to accumulate as proof that selectivity of host plants is an important factor in the establishment of new races of fungi. This selective influence may be in the direction of either greater or less pathogenicity. Several workers have demonstrated the influence of host selectivity in separating races of *Tilletia* spp. from mixed populations of races. According to Dillon-Weston (48) certain resistant varieties of wheat were rendered completely susceptible by reinoculating them with bunt spores that came directly from these varieties. Bressman (12) obtained similar results with bunt and concluded that new virulent races could be obtained by this method. Likewise, Mourashkinsky (103) found that the passage of certain races of *T. caries* and *T. foetida* for five consecutive years through the same variety of wheat, increased considerably their virulence on that variety. Holton (73) found that highly susceptible varieties of wheat, like Hybrid 128 tended to promote the establishment of weakly pathogenic races with a narrow host range, while resistant varieties promoted the establishment of races with high virulence and a wide host range. Holton states that races of high virulence in nature and wide host range are most populous in regions where new smut-resistant varieties are frequently introduced.

Obviously, the degree to which the pathogenicity of a race can be changed depends on its genetic make-up. Bever (7) observed no increase in virulence on resistant varieties when pure races of *Tilletia* were used, while the pathogenicity of a hybrid population greatly increased with repeated passage through certain varieties. Vielwerth (153) concluded that the pathogenicity of a bunt population gradually decreases when it is constantly propagated on moderately susceptible wheat varieties.

The success of selecting races or biotypes of smuts from a mixed population depends on the heterogenicity of the original material (71, 72, 74, 75, 143). Attempts to alter the pathogenicity of collections of smut by passing them through resistant varieties may be successful or unsuccessful according to the findings of Tervet (145) and others (123, 151). In some cases Tervet was able to increase the amount of oats smuts (*U. avenae* and *U. kolleri*) on

Black Mesdag by using inoculum from that variety, whereas no success was obtained when inoculum was taken from other varieties. Bever obtained similar results with *T. foetida* (7). Likewise, Sampson & Western (129) were unable to increase the pathogenicity of three races of oat smut (*U. kolleri*) over a ten-year period. Selaries (131) obtained no increase in virulence by the propagation of bunt on resistant or susceptible varieties.

The influence of resistant hosts in the selection of new races also has been reported for other smuts. Reed (120), Nicolaisen (110, 111), and Sampson & Western (129), were able to increase the virulence of certain races of *U. avenae* and *U. kolleri* (loose and covered smut) by successive passages through resistant varieties of oats. Likewise, Roemer & Kamlah (125) were able to increase the virulence of loose smut of wheat (*U. tritici*). Bever's result indicates that new varieties of wheat shift the prevalence of different physiologic races of *U. tritici* (8).

Tervet (146) found that the reaction of testers for oat smut in different years to *U. avenae* was inconsistent. This was explained, at least in part, on seed lots of a given variety grown either in different years or in different geographical regions in the same year. The quality of seed also was found to be an important factor in varietal reaction (147).

The variety on which the race is grown tends to perpetuate those biotypes to which it is most susceptible. By this method it is possible to separate not only different species, but also races in mixtures used for inoculations. Thus, Flor (55) separated a race of *T. caries* from a mixed population of *T. caries* and *T. foetida* by the selective influence of several varieties of wheat. Holton (72) and Rodenhiser & Holton (123, 124) obtained similar results by using known mixtures of races.

These results indicate that the virulence of a microorganism may be altered tremendously when it is passed through a living host by selecting those biotypes of given species that can attack a hitherto resistant variety. Holton (72) proved this by crossing races of *T. caries* and *T. foetida*. New virulent races were produced whenever the hybrid inoculum was increased on a relatively resistant variety (72, 75, 124). He concluded that the selective influence of a host variety is a highly significant factor in establishing new races of bunt. He also made crosses between white and black races of *U. avenae* and obtained similar results. The nature of this selective influence appears to depend largely upon the degree of resistance or susceptibility of hosts. Hitherto resistant hosts promote the development of virulent biotypes because only the most virulent lines can attack these varieties. There is no reason why the susceptible varieties should select out virulent races unless these races are produced in greater abundance than the more common races. Therefore, the selective effect of host varieties in providing a source of inoculum from year to year may have an important role on the severity of the attack. For example, a population of chlamydospores from Ulka wheat would provide inoculum whose pathogenic potentialities could differ strikingly from a population of spores from Ridit or Oro.

The persistence of physiological races of smut after many passages through host plants is a remarkable phenomenon. In most cases, where the original inoculum was obtained from a field collection, it consisted of millions, if not billions of chlamydospores in a heterozygous condition. Even after the races have been "purified" by screening through certain hosts, the chlamydospores are not always exactly alike. In spite of this, Reed (120), Rodenhiser & Holton (123), and others, have found that the pathogenicity of many races of smut remain stable over long periods.

This pathogenic stability of a physiologic race of smut is even more unique when one considers that the smuts pass through a sexual generation each time chlamydospores are formed. The parasitic mycelium of smuts is dicaryotic and the chlamydospores are diploid, the result of fusion of nuclei of the opposite sex. When the chlamydospores germinate, segregation and recombination occur, so that a progeny of a single chlamydospore in one generation may be quite diverse genetically. Therefore, races of smut normally consist of many biotypes which are closely related and for practical purposes behave with relative consistency in successive generations of differential hosts. However, new races can be derived from established races by the selective influence of a host or by selecting compatible biotypes from artificial cultures (74, 110, 152). Many workers (62, 72, 111) have demonstrated that new parasitic races of smuts can readily be obtained simply by pairing two lines of the opposite sex, even from the same chlamydospore.

A race of smut then, is usually a collection of chlamydospores in a heterozygous condition that behave with relative constancy on a set of differential hosts in successive generations. Classification of smuts into races suffices for practical procedure, but not for basic studies on adaptation.

Late blight.—The development of new parasitic races of late blight (*P. infestans*) that can attack hitherto resistant varieties of potatoes has never been adequately explained. Reddick & Mills (118), Mills (100) and others reported that by serial passage of *P. infestans* through a medium susceptible or resistant potato variety, they were able to increase the virulence of a single-zoospore culture to the extent that it attacked several varieties of potatoes which were immune or highly resistant to the original culture. Later, Reddick (116, 117), Reddick & Peterson (119), and Mills & Peterson (101) concluded that the virulence of *P. infestans* could be increased by serial passage of the fungus on senile foliage of resistant potatoes. De Bruyn (42, 43) repeated this work and concluded that more virulent races developed from the repeated passage of *P. infestans* through tubers and potato foliage. Several workers (10, 105, 106, 142, 154) have been unable to find evidence of adaptation.

It has been suggested that the part or age of the host from which the inoculum was taken might play a role in adaptation. Thurston & Eide in 1953 (148), were unable to demonstrate adaptation of *P. infestans* by reinoculation of the same or other resistant clones with sporangia produced on atypical flecks on resistant plants. Several investigators reported that races

virulent on tomato foliage also were capable of attacking potato foliage. Mills (100) in 1940 increased the virulence of a potato race by its serial passage through a tomato plant. This culture, after several passages through the tomato, which it originally attacked only weakly, became as virulent as the normal tomato race. De Bruyn (43) obtained similar results. It is noteworthy that virulent isolates on tomato foliage have been obtained from naturally infected potato tubers.

De Bruyn (42) and Reddick & Mills (118) emphasized the plasticity of *P. infestans*, while others found that races, once established, are relatively stable with respect to pathogenicity. Some workers have stated that a high degree of virulence in an isolate will be maintained even after continual passage through a susceptible host (100, 101, 119). According to Mills (100), once the pathogenicity was raised to a certain level, it remained there, even after 20 passages on any host. De Bruyn (42, 43) found that there was a gradual change of infective capacity of the potato isolate towards tomatoes and that these newly gained characters may be lost after a repassage through potato. Therefore, pathogenicity of an isolate invariably depends on the final host passages irrespective of their origin or previous treatment.

Müller (107) suggested that "new races" may be present in original inoculum and may coexist without manifesting themselves until a suitable differential host is presented. Thurston & Eide (148) found that isolates of *P. infestans* from field collections sometimes produced susceptible and resistant types of infection on young potato plants of resistant clones. Isolates made from the susceptible types of lesions on resistant varieties proved to be a new parasitic race, distinct from the race predominant on the leaves. They postulated that races existed in small quantities in biological populations of isolates from which they came; yet did not exclude the possibility of mutation. Recently Castronova, Thurston & Eide (23) have emphasized the differences in infectivity and aggressiveness among races of *P. infestans* in the field on different varieties in mixed population. This may play an important role in the survival of races in the field.

Other fungi.—There is often a decrease in virulence of fungi when they are grown in an artificial medium over a period of years. Sometimes these changes appear to be gradual; yet, frequently they are sudden and not necessarily associated with their duration in culture. Occasionally they appear to be cyclic in nature. Leonian (90) found that isolates of *F. moniliforme* were extremely variable in virulence. In one test, certain isolates infected the host, whereas at another time, they did not. He considered this a normal cyclic variability for the species.

Christensen (28), like Leonian, has observed that certain isolates of *F. moniliforme* are very unstable, mutation being common, but he has never encountered cyclic changes in parasitism. When changes in virulence did occur they were always sudden and relatively stable and apparently genetic in nature. Similar results also were obtained with *F. lini* and *F. graminearum*. Isolates of several fungi have maintained their virulence from 10 to 30 years, others for only one generation on a culture medium.

Burkholder (18) reported that the virulence of *F. martii phaseoli*, after growing six years in culture, had been materially lowered. When this organism was passed through the host two successive times its former virulence was restored.

Müller (104) isolated *Gloeosporium fructigenum* from *Phaseolus multiflorus* and then reinoculated many varieties of bean plants. He secured good infection on only one plant, from which single-spore isolations were made. Inoculations were then made on several other hosts. The isolates not only differed in virulence on different hosts but the pathogenicity of an isolate for a particular host could be increased by passage through that host. Thus, the isolate grown on apple was much more virulent on that host than the isolate grown on bean plants and vice versa.

Robert (122) found that single-conidial isolates of *H. turcicum* differed in their ability to infect corn from year to year. This was equally true whether the isolates were maintained on potato-dextrose agar or were passed through a susceptible variety of corn. In some instances, the virulence of isolates was increased by passages through the host; in others, there was a decrease in virulence. Similar results also were obtained by growing the isolates on potato-dextrose agar.

Some species of *Helminthosporium* appear to be heterocaryotic, whereas others appear to be monocaryotic (30, 59, 122, 132). Moreover, mutations are very common in many species of *Helminthosporium*. Therefore, the changes in virulence could be expressed on the basis of nuclear dissociation and mutation.

For many years Christensen and his associates have studied the effects of passing a pathogen through a living host. *Helminthosporium* species have been used extensively in these studies (29, 30, 33). In one case an isolate of *H. sativum*, which had been single-spored many times and which had grown for 28 years continually on artificial media, was passed through the wheat host 10 successive times. Wheat seedlings, free from fungi and bacteria, growing in large test tubes, were inoculated with a single spore of *H. sativum* by means of a fine needle. Then, when the spores were produced they were isolated and single spores were again inoculated directly into a new set of wheat seedlings. The culture that had passed through the host 10 times was no more virulent than the control culture which had been growing for at least 28 years on artificial substrates.

Similar results were obtained by passing monosporous isolates of *D. zeae*, *F. graminearum*, *F. lini*, and *C. linicola* through their respective hosts (28). Keitt & Langford (88) passed three haploid isolates of *V. inaequalis* through apple leaves four successive times without altering their virulence. Likewise, Bailey (5) was unable to change the virulence of *C. fulvum* although it was passed through the host 18 successive generations. These results indicate that the virulence of a genetically pure culture that has been on artificial substrates, for varying periods of time, cannot be altered perceptibly by passage through the host. Since mutation, heterocaryosis and nuclear dissociation may occur in pathogens in host plants, changes in virulence can

be expected occasionally by repeated passage through the host. Thus, Chilton (25) found two variants of *C. distructivum* in a population of 986 spores, while Christensen & Schneider (33) found the frequency of mutation in *H. sativum* on hosts to be 1:2900.

Bacteria.—There are many reports that pathogenic bacteria become attenuated when grown on artificial culture media. The virulence of these isolates can often be restored by passing them through appropriate hosts. The mechanism by which the host changes the isolates is not definitely known, but it is often referred to as "adaptation."

Hendrickson, Baldwin & Riker (67) reported changes in the physiological behavior of *Agrobacterium tumefaciens* by passing it through the tomato plant. Allen & Baldwin (2) proved that infectivity of *Rhizobium* was materially altered by repeated passage through appropriate plants. Sharp (133) and Elcock (50) reported cultural and pathogenic changes in *Phytomonas beticola* by passage through susceptible soybean plants, and Elcock (50) noted changes in cultural characters of *P. phaseoli sojense* after a passage through a host.

The fire blight organism, *E. amylovora*, is relatively unstable and variable in culture and also in nature. Hildebrand (68) found some isolates remained consistently high in virulence whereas others lost their virulence. In pathogenicity tests, isolates derived from single cells were less variable than those derived from the parental isolates which were originally obtained from a single colony. The passage of weakly virulent isolates through susceptible host tissue usually restored their virulence. Since the isolates differed not only in pathogenicity, cultural characters, and physiology, but also in morphology, the isolates apparently represented different biotypes of *E. amylovora*.

Ivanhoff (78) made inoculations in the greenhouse and field with strains of *P. stewartii*, the causal agents of bacterial wilt of corn, derived from single cells and found that they infect sorghum, indian grass, yellow foxtail, german and common millet. He observed no change in the pathogenicity of the bacteria as a result of passage through these plants. No mention, however, was made of the number of passages through the respective hosts. Later Ivanhoff *et al.* (79) observed marked differences among isolates, particularly in physiologic and morphologic characteristics of colonies.

One of the most comprehensive studies on adaptation of phytopathogenic bacteria to their hosts has been made by Wellhausen (164), Lincoln (92), and McNew (98). They proved that successive passages of an isolate of *P. stewartii* through a susceptible host decreased its virulence while passage through a resistant host increased its virulence. The virulence of *P. stewartii* could again be changed by reversing the host through which the isolates were passed. For instance, Lincoln (92) decreased the virulence of 13 single-celled isolates of *P. stewartii* by passage through susceptible lines of corn, whereas the isolates maintained their virulence by passage through resistant lines of corn.

According to Wellhausen (164) successive passages of *P. stewartii* through a susceptible or resistant strain of teosinte affected its virulence similar to passage through corn. Also, successive passages of the isolates through other grass hosts reduced their virulence for corn, although there was a tendency for isolates to become more aggressive for the respective grass hosts through which they were passed.

There also was a tendency for the isolates to reach an equilibrium on a given host, beyond which further passage had no effect. Thus, virulence could not be increased beyond a certain level in resistant varieties of corn nor could it be decreased beyond a certain level in a susceptible host. The virulence of some lines of *P. stewartii* was more stable than that of others. One isolate, with low virulence, failed to revert to high virulence after 13 passages through the resistant host, whereas others readily acquired virulence (92).

Lincoln (92), Wellhausen (164), and McNew (98, 99) concluded that the change in virulence of an isolate was associated with the genetic make-up of the host and genetic variation in the populations of bacteria. Quantitative proof of this was obtained by inoculating susceptible and resistant hosts with a known mixture of virulent and weakly virulent bacteria. There was always a differential selection for virulent lines in a resistant host. Mutation apparently occurred commonly in *P. stewartii* in culture and perhaps also in the host.

Lincoln (92) and McNew (99) isolated many variants from stock cultures that differed not only in cultural characters but also in virulence. Because of the rapid rate of reproduction in a growing population of bacteria, considerable variation in populations of bacteria can occur in relatively short periods of time, even if the rate of mutation is very low. Thus, Lincoln calculated the frequency of mutation in *P. stewartii* to range from 1 in 20,000 to 1 in 800,000 individual cells.

Apparently mutation and natural selection are two important agencies in bringing about alteration in virulence of *P. stewartii* during its sojourn on the host. Since, in these tests, the host plant was not inoculated with a single bacterial cell, but rather with a large population, it cannot be stated definitely that the changes did not occur in broth cultures before inoculations were made. In any case, the proportion of virulent cells in a population was increased materially by passages through the resistant host. Reisolation proved that there was a mixture of biotypes present.

McNew (99) was not only able to restore the virulence of attenuated isolates of *P. stewartii* by a sojourn on a host but he also restored it by growing the isolates on a synthetic medium containing no organic nitrogen. As there was a high correlation between virulence and the ability of an isolate to use inorganic nitrogen, a medium free from organic nitrogen tended to select variants capable of utilizing inorganic nitrogen. Therefore, it was possible to select highly virulent lines on this medium and to eliminate weakly virulent lines. A phenomenon similar to this may account for the change in

virulence on passage through the host. In any case, weakly pathogenic isolates, when induced to grow on a medium containing inorganic nitrogen, remained highly virulent.

Changes in virulence were not caused by a physiological stimulus of the pathogen, but rather a mechanical selection of variants. These changes in virulence of bacteria are very similar to those obtained by passing fungi, like smuts, through susceptible and resistant hosts.

CONCLUSION

The material presented clearly indicates that the virulence of a pathogen may be maintained, enhanced, or decreased by a sojourn on a host. That "adaptation" of fungi and bacteria can occur by successive passages through a host is an established fact. In most cases, however, changes do not involve adaptation in the strict sense but they are genetic changes, which are associated with mutation, hybridization, or heterocaryosis and dissociation of nuclei, the host acting as a selective agent.

Many observations on adaptation involving host-parasite relationships have been made in the greenhouse and in the field. Under such conditions, the possibility of contamination by other pathogenic races or biotypes cannot be disregarded. In some experiments, inoculum consisted either of many hyphae or of a large population of spores, or both, hence the possibility of genetic mixtures in the inoculum should be considered. Genetic variation, resulting from interbreeding among biotypes, contributes to the dynamic nature of race populations which frequently give rise to biotypes parasitic on new varieties. A host variety often determines the race population by its selective influence on hybrid inoculum.

Extensive studies on mutations in culture have established principles that are important in interpreting apparent adaptation in fungi. Mutants frequently cause mixed populations in lines which were considered as stable biotypes. Moreover, cultures, that appear to be single biotypes may actually contain many different biotypes which are not evident without special methods of detection. That mutations frequently occur in cultural and many physiologic characters, including virulence, has been demonstrated repeatedly for many organisms. Thus, with the ever increasing number of studies on the variation and variability in microorganisms, it is becoming more apparent that genetic purity of isolates is a concept, not a reality.

However, a number of studies have been made which indicate that a readjustment of the metabolic systems of plant pathogens in various environments has affected their pathogenicity and virulence without involving a nuclear change. In certain cases where a change in light, temperature, host nutrition, or age has markedly changed the interaction between host and parasite, it is difficult to ascribe this change to either increased virulence of the pathogen or to increased susceptibility of the host. These changes in disease expression are probably a result of environmental changes affecting

both the host and the parasite. Yet, it is possible that alterations in the nutritive environment provided the parasite in the host, may stimulate adaptive mechanisms in the parasite which affect its virulence.

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PHYSIOLOGY OF ALKALOIDS[1]

By K. Mothes[2]

Institut für Kulturpflanzenforschung Gatersleben, Kr. Aschersleben, Germany

The experimental study of the physiology of alkaloids is relatively recent although interest in the nature and metabolism of these substances can be traced back to the middle of the last century. With the discovery of the synthesis of some important alkaloids in the root, this experimental treatment initiated a new development. A number of summary accounts of the investigations in the early period of the alkaloid inquiry exist [Dawson (44, 45); Hegnauer (92); Iljin (115); James (122); Mothes (171)]. Investigations described in those accounts are mentioned here only in so far as they appear necessary for a complete description.

Also, recently the chemistry of the alkaloids has been considerably elucidated. Summary accounts may be found in Henry (97), Dawson (46), Manske & Holmes (155), Boit (11) and Guggenheim (79). A comprehensive review of the literature on the paper chromatography of alkaloids is given by Bräuniger (19).

Formation of Alkaloids in Roots

In former investigations, it was surprising to find that fully-developed leaves of alkaloid plants increased their alkaloid content when attached to the plant, whereas, when detached, they showed no alkaloid synthesis under very different conditions of nutrition, exposure to light, etc. [Mothes (168)]. Later Dawson (39) reported that excised segments of tobacco stems are able to develop lateral branches and callus which are both nicotine-free. But if excised leaves have been rooted in humid sand, they accumulate nicotine in large amounts.

Nath (187) and Bernhardini (9) showed that nicotine decreases greatly in tobacco scions on tomato stocks. Reciprocally, grafts of tomato upon tobacco contain nicotine in both stock and scion. However, these investigations remained unknown for some time. Hasegawa (85) devised similar experiments and obtained the same result. In the years 1939 to 1941, a number of Russian publications appeared, reporting the results of grafting experiments with alkaloidal plants [Evtushenko (62); Kerkis & Pigulevskaja (131); Kuzmenko & Tikhvinskaja (147); Schmuck, Kostoff & Borozdina (222); Schmuck, Smirnov & Iljin (223)]. Several of these pioneers of alkaloid investigation were so strongly influenced by the doctrinal definitions of the publications of Michurin and Timiriazew that they did not draw the obvious conclusion that the root is the locus for the production of alkaloids. Moshkov

[1] The survey of the literature pertaining to this review was concluded in October, 1954.

[2] I thank my wife, Dr. Hilda Mothes, for the translation into English.

& Smirnova (166) reciprocally grafted lupines rich or poor in alkaloids and found that the proportion of alkaloid of the scion varied according to the stock. They wrote:

> In undertaking such experiments we started from the fact that plant metabolism is greatly dependent upon the synthetic activity of green leaves by whose means the plant, according to the lively expression of Timiriazew, is performing its cosmic rôle. We are also guided by the indications of Michurin to the significance of leaves in grafting. To be sure, it can hardly be doubted that synthesis of alkaloids is also bound up with the life activity of the leaves. . . .

Although Kuzmenko & Tikhvinskaja (147), grafting *Nicotiana glauca* with *N. rustica* and *N. tabacum*, had correctly determined the migration of the alkaloids from the stock to the scion, Schmuck and collaborators (222, 223) considered the modifications of the alkaloid character of the scion not as the result of a translocation of alkaloids, but as a total transformation of the metabolism of the scion under the influence of the stock. These concepts have influenced the writings of Glustschenko (73) which appeared in 1942. Meanwhile, working independently of each other, Dawson (39), Hieke (99), Mothes & Hieke (178), and Pal & Nath (194), became convinced that the alkaloid content of the scion was decisively determined by the alkaloid production in the root as well as by the migration and accumulation of the alkaloids at secondary loci, particularly the leaves.

A great number of grafting experiments done by others corroborated these results. Cromwell (35) grafted tomato with *Atropa* and Peacock, Leyerle & Dawson (196) grafted *Datura stramonium* with tobacco and tomato. Iljin (115), too, reports that he succeeded in demonstrating that nicotine is formed in the root.

We should not forget that in the beginning of this period of investigations it was quite all right to say [Kuzmenko & Tikhvinskaja (147)] that substances in grafted plants flow from stock to scion and from scion to stock. These substances might be of two different kinds. It is possible that molecules from which alkaloids result migrate from one partner to another. On the other hand, they may conceivably be substances which determine the direction of the synthesis of the alkaloids, nicotine and anabasine. The questions raised here are still important. Of primary importance then, however, was the question of whether the root itself could really produce alkaloids. Some of the conclusive proof came first with the discovery of alkaloid in the bleeding sap of alkaloidal plants [Dawson (38a, 39); Hieke (99); cp. Mothes (170)]. From this observation was adduced the probability of the translocation of alkaloids from the root to the scion. The second fact to be established was that nicotine is not equally spread within the whole scion of tomato graft on tobacco, but is concentrated chiefly in one sector [Dawson (39)]. This was difficult to reconcile with the synthesis of nicotine in the tomato scion itself. The third and most convincing proof came from sterile cultivation of excised roots. Thus, alkaloid in isolated roots was found by

Dawson (38a, 40) in tobacco; Peacock, Leyerle, & Dawson (196) in *D. stramonium;* Mothes & Kretschmer (179) in *Lupinus;* Telle & Gautheret (244) in root tissue of *Hyoscyamus niger;* Uffelie (249), in *H. aureus* and *D. stramonium;* Moreno (164); and Manrique & Moreno (154) in *Atropa.* In all these cases, the quantity of alkaloid in one root increased remarkably. Doubtlessly, alkaloid in considerable quantities was produced and was secreted, too, to some extent, into the liquid medium [e.g., Dawson (40)]. However, from the published reports mentioned above, it is difficult to decide whether considerably more alkaloid was accumulated in excised roots than in those attached to the plant which may continually deliver alkaloid to the scion. At the present time, no satisfying comparison of alkaloid content of attached and detached roots has been made.

There are still other indications of the special role of the root in alkaloid synthesis. Scions of alkaloidal plants grown on alkaloid-free stems produce leaves virtually free of alkaloid. If such leaves are isolated in water, alkaloid does not appear. When, however, the development of adventitious roots is promoted, alkaloids appear [Dawson (38a), Iljin (113)]. Laschuk (148) reports that the development of embryonic roots at the grafting site of a scion suffices to provide the scion with alkaloid. He not only concludes that the production of alkaloids takes place in the tips of the roots, but also that the development and growth of the root is a requirement for this synthesis. However, it is not fully proved that the production of alkaloid is definitely associated with the growth of the roots. Thus, Trapp (245) has reported on isolated nicotine-free tobacco leaves which are able to produce nicotine-free adventitious roots in N-free nutrient solution. This statement requires verification.

The investigations of Wallebroek (257) on the germination of *L. luteus* indicate that the root is the specially favoured locus of alkaloid synthesis. In the first three days of germination, he observed a tenfold alkaloid increase in root and plumule, but a decrease in the cotyledons. Cromwell & Rennie (36) discovered that in *Beta vulgaris* and other plants glycine betaine is likewise produced in the root and transported into the scion system enriching the axis and leaves. A problem of particular interest is the existence of alkaloids in the latex whose physiology is little understood even today. Wegner (260) found that morphine first appears in the root of *Papaver somniferum* but that, in the course of development, the center of morphine distribution rises continuously until, at the time of ripening, the capsules contain most of the morphine (78.7 per cent). In the lower organs, moreover, morphine decreases, not only relatively to the ratio of dry weight, but also absolutely so that one might be inclined to assume that it migrates upward. But these investigations do not yet prove the synthesis of morphine in the root.

Further indications are found in the investigations of Cromwell (33) on the existence of berberine in *Berberis darwinii.* This alkaloid, which is strongly adsorbed to the cell wall, appears to have its highest concentration

in the root. The alkaloid content distinctly increases from the younger twigs to the mainstem, from the top to the base of the stem, and to the root.

Other indications supporting the theory of alkaloid synthesis in the root were obtained by microchemical investigations. Chazé (31) found nicotine in the apices. James (119) found similar results in the radicles of *Atropa* and *Datura*. Solanaceae are particularly qualified for such studies, as their embryos and endosperms are consistently free of alkaloid. But in all microchemical reactions, the limited specificity of the reagents must be taken into consideration. The same is true for the fluorescence-optical method after treating the tissues with bromine cyanide, as applied by Schmid (218) and Schmid & Serrano (219). For, in this case, all pyridines show fluorescence, e.g., niacin. These Swiss authors conclude on the basis of their studies with *N. tabacum* and *N. rustica* that nicotine synthesis in the embryo proceeds through two phases: first at the expense of the proteins near the aleurone grains found in all parts of the germ; later only in the root, probably as a by-product of albumen synthesis. This second phase is slow in coming into action.

Codounis (32), reports similar results based on the polarization microscopic test for alkaloid picrates which crystallize *in situ* after the addition of picric acid. He found alkaloid first in the hypocotyl of four-day-old tobacco embryos. During further development, too, the maximum alkaloid content was found there. On about the eleventh day after germination, the alkaloid concentration falls, rising again after the development of the second leaf. Fardy, Cuzin & Schwartz (63) correct these assertions. After 36 to 48 hr., they found nicotine in the meristem of the radicle and only later in the hypocotyl. Alkaloid seems to be transported from the root into the axis. Of course, we must include the possibility that translocation may occur as quickly as the synthesis itself. Engelbrecht (55, 56) has showed the capacity of the hypocotyl for storing soluble nitrogen compounds, especially amides and allantoine.

Alkaloid Synthesis in the Shoot

The investigations with embryos raised the question whether alkaloids are produced in the root only or whether parts of the shoot are also able to do so. After the first results of modern grafting experiments were known, opinions changed. Aside from the Russian authors who first denied the existence of synthesis in the root and who considered it possible only in the shoot (under the influence of the root, however) two opposing points of view arose: the one (at least for several alkaloids) asserted that the root was the only site of production; the other defined the root as the "*Hauptbildungsstätte*" [Mothes & Hieke (178)]. In the early investigations on germination, we observed the difficulty of deciding whether an alkaloid content was primary or secondary. James (119) found alkaloid microchemically, not only in the meristem of the root but in the shoot meristem as well, and in the apices of all buds. And he indicates (122) that not only primary but also

secondary meristems and, above all, tissues in which nuclear divisions take place are loci of special alkaloid concentration, whereas the root-cap does not show a reaction. However, it remains uncertain whether these meristems are loci of special accumulation of alkaloids or of the synthesis itself.

After Mothes, in 1928, had contended that a slight nicotine synthesis existed within young isolated tobacco leaves, Cromwell (35) reported alkaloid synthesis in detached leaves, and later, and more important, James (118, 119, 121) found a slight alkaloid synthesis in detached young leaves of *Atropa* if maintained on distilled water in the dark to the stage at which the leaves become noticeably yellow. He argued that proteolysis and the formation of amino acids are the causative factors of alkaloid synthesis, the exact extent of which may be difficult to determine since autolysis proceeds quickly. James also wrote about the synthesis of alkaloids at the cost of special amino acids in detached leaves [cp. (121)].

Hofstra (106), however, does not find alkaloid formation in isolated tobacco leaves, and it seems necessary once more to investigate the whole complex of problems with new methods, particularly as James (118) noticed that neither older *Atropa* leaves nor those which come from *Atropa* scions grown on tomato stock can synthesize alkaloids. Thus, important differences seem to exist between grafted and ungrafted *Atropa* as to their biosynthetic capacities.

The differentiation of root and shoot as organs of different chemical potency is a central problem of the physiology of development. Since shoots and parts of shoots, as well as leaves, are capable of regenerating roots, the capacity for root development must latently exist in the shoot. The question is, therefore, whether or not the shoot develops this potential because of internal or external factors.

In this direction, grafting experiments produced new evidence. In the early stages of the new investigations, only traces of alkaloid were found in scions of normally alkaloid-containing plants grown on alkaloid-free roots. One took for granted that these scions were practically devoid of alkaloid because the minute quantities found might well have been introduced by the grafted scion.

Mothes & Romeike (180), and Romeike (211) investigated these problems more thoroughly in 1951 and 1953, respectively. Repeatedly, they excised *Atropa* scions growing on tomato roots so that only a short piece of the *Atropa* shoot remained, which then produced new branches. In cutting off most of the scion practically all alkaloid was removed from the scion. Nevertheless, in the freshly developing *Atropa* branches, alkaloid continued to be found. It reacted mydriatically and showed the Vitali reaction. This had to be considered proof of alkaloid synthesis in the scion, for mydriatic alkaloids could never be demonstrated in tomatoes. Also, in grafting *Datura* on *Cyphomandra*, Romeike (211) always found mydriatic alkaloids in the younger leaves of the scion but not in the older ones. She, too, observed a certain dependence of alkaloid synthesis in the scion on the type of stock.

Different tomato strains used as stock produced different quantities of alkaloid in the scion. Tobacco stocks especially produce tropane alkaloids. Hegnauer (87, 88), too, in grafting *Datura* on tomato, found hyoscyamine in all parts, especially in flowers, young pericarps, and young seeds.

Maskovcev & Sirotenko (158) have tried to eliminate the source of error arising from the fact that the scions themselves contain a certain quantity of alkaloid. These workers therefore grafted young tobacco embryos almost entirely free of nicotine on tomatoes. The tobacco scions, maturing slowly, contained 21 mg. of nicotine in the leaves alone. The interpretation is that the alkaloid content had risen about 100,000-fold. However, these investigations have two disadvantages. First, it was established that tomato itself is able to form nicotine (Wahl). Therefore it seems problematic whether the nicotine found in the tobacco scion is produced there or whether it migrates from the tomato stock. Furthermore, our own experiments with embryo grafting have shown that, very easily and quite frequently, roots are formed by the scion which may grow inside the stem of the stock and are not necessarily visible to the observer. Such roots are always a serious source of error. It may be thought that a tomato plant would never form as much nicotine as the above tobacco scion contained, but that does not necessarily mean that the alkaloid was formed within the scion. It is possible that the tomato plant, under normal conditions, forms more nicotine in the root than was found in the tobacco scion experiment, and which is destroyed in the tomato scion, but is preserved in the tobacco scion. As shown below, no positive statements about a more extensive degradation of alkaloids in the shoot can be made.

If we assume that alkaloid synthesis in the shoot is established, there still remain questions to be answered. Why do we always find alkaloid in the youngest parts of the scion only? This is the more surprising since, at the beginning of the grafting experiment, it was found that the alkaloid taken in by the scion is located chiefly in its lower leaves. The distribution of alkaloids in the scion on alkaloid-free stock is reversed from that in the ungrafted shoot. In the latter, the concentration diminishes from base to top; in the former it increases from base to top. These facts also favour the theory of an alkaloid synthesis in the youngest portions of the shoot. The second question is: why does a scion grown on a foreign alkaloid-free root always form so little alkaloid? Excepting the possibility that the original root might be essential for alkaloid formation in the scion, possibly by providing specific precursors of the alkaloids in question, there must also be considered much simpler reasons for the superiority of the root in the process of alkaloid synthesis.

In the publications of Mothes & Engelbrecht (176, 177) and Engelbrecht (55) it was shown that in *Acer*, Borraginaceae, and also in *Phaseolus*, the roots are the preferred loci of allantoine synthesis. A great deal of the inorganic nitrogen infiltrating the roots, as well as the NH_3 resulting from proteolysis, changes to allantoine. This substance, until now implicated only

in nucleic acid metabolism, can constitute 70 per cent of the total N in roots. It is a neutral storing and translocation form of nitrogen. Wolffgang & Mothes (267), investigating bleeding saps, demonstrated further that *Alnus* root chiefly accumulates citrulline, and Reuter & Wolffgang (206) found that, for all indigenous Betulaceae, this accumulation of citrulline is as family-specific as that of allantoine for the Borraginaceae, etc.

Thus, it was obvious that the process of primary assimilation of inorganic nitrogen might be responsible for the appearance of the alkaloids. Attempts were made, therefore, to shift this primary assimilation to the leaves by total elimination of the root. For this purpose, leaves of Solanaceae which received no nitrogen at all from the soil, were sprayed regularly with nitrogen-containing solutions [Mothes & Trefftz (184)]. Such sprays, especially those containing urea have been frequently used for the purpose of providing additional nitrogen to plants. However, the spray, in this case, which was the only source of nitrogen, was unsatisfactory since, in the Solanaceae, urea is quickly transformed into NH_3 and thus acts as a poison. $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2HPO_4$ were equally unsatisfactory. In comparative experiments, NH_4NO_3 was the most successful. Frequent spraying results in a totally normal development which does not differ in any respect from that of the control plant which was nourished only by the root. *Nicotiana*, *Atropa*, and *Datura* plants did not differ in principle whether nourished by the leaf or by the root. They contained the same quantity of alkaloid. Were the alkaloids in both cases principally produced in the root?

If plants nourished by the leaf and those nourished by the root are cut off at the base of the shoot, bleeding sap may be squeezed out at the section. Plants are able to bleed for several weeks and secrete large quantities of alkaloid. The alkaloid concentration is approximatively the same, and is independent of the preceding nitrogen nutrition by leaf or root. Thus, those plants which do not receive nitrogen from external sources also transport nicotine in an upward direction. Still more conclusive is another experiment. If *Atropa* is grafted on *Lycopersicon*, the scion contains only very small quantities of mydriatic alkaloids. If this scion is nourished with nitrogen from its own leaves only, and the root of the tomato stock receives no inorganic nitrogen from outside, the scion contains as little alkaloid as the control plants which are nourished by the root. From this it must be concluded also that in the case of nitrogen absorption by leaves, alkaloid is substantially synthesized in the root [Mothes *et al.* (185)].

It might be precipitate to assume, on the basis of these experiments, that it is not possible to shift alkaloid synthesis from the root into the shoot, for all the experiments described here have one common defect. It appears that only the middle-aged or the older leaves are able to absorb the sprayed nitrogen solution; the youngest do so only with difficulty or not at all. All hitherto existing results, however, attribute the capacity of synthesizing alkaloid only to the youngest leaves. In the root, too, only the youngest part is capable of synthesis.

It would be very interesting if we might succeed in demonstrating alkaloid production by a culture of rootless, isolated shoot parts or shoot tissues. Such cultivation, under sterile conditions, has been done more than once [see Boysen Jensen (16)]. But nothing is yet known about alkaloid synthesis under such conditions. The experiments are very difficult to do because large shoot segments totally regenerate in sterile culture, which means they also produce roots. Thus, the positive proof of alkaloid synthesis loses its demonstrative force.

The question of the assumption that alkaloid synthesis proceeds from the shoot gains in importance because of the exceptions to the rule that the root is the *Hauptbildungsstätte* of the alkaloids. Shoot synthesis in measurable quantities has been positively noted for steroid alkaloids of the solanidine type. Recent literature may be found in Street, Kenyon & Watson (242) and Prokoschev and collaborators (198). One might be tempted to exclude this observation, because solanidines have an exceptional position among the alkaloids. Structurally, as well as in their binding to sugar, they show a closer relation to saponines than to other alkaloids. But there is another and more significant "exception"—anabasine. Schmuck, Kostoff & Borozdina (222) in 1939, and Schmuck, Smirnov & Iljin (223) in 1941, recognized the exceptional position of anabasine in grafts between *N. glauca* on the one hand and *N. tabacum* or *Lycopersicon* on the other, although they certainly did not interpret it correctly. These older studies are filled with uncertainty, based on the failure to separate anabasine from nornicotine. Dawson in 1944 (41) fully solved the anabasine problem and was essentially corroborated by Iljin (115). The decisive experiments are grafts of *N. glauca* on roots of tomato or tobacco. In both cases anabasine appears in the *N. glauca* scion; in the second case, nornicotine also appears. Thus, the alkaloid content of the scion does not react according to the stock but shows its own qualities which did not arise from the different stocks. Dawson concluded that anabasine can also be produced in the leaves. It may be questioned whether, in addition to a total new formation of anabasine, a transformation of translocated nicotine into anabasine was possible [Iljin (113, 115, 116)]. In this reviewer's opinion, strong evidence for such a hypothesis is lacking.

To establish proof is of great importance because tomato, too, is able to produce nicotine, although normally only in inconsiderable amounts. Without doubt, there is still a small missing link in the chain of arguments. For, if we suppose that anabasine can actually be produced from nicotine within the *N. glauca* leaf and in addition, suppose that tomato root produces more nicotine than is realized from our analysis of the tomato plant (nicotine may be degraded again in the tomato shoot), a measurable result might be obtained without accepting the hypothesis of an anabasine synthesis in the *N. glauca* shoot. In this case it is important that anabasine is to be found in other *Nicotiana* species, too, albeit in small quantities ("Nebenalkaloid"). In our own continuing experiments we have not observed that such tobacco species, even as scions upon tomato root, contain anabasine.

Of special interest are the investigations of Jackson & Rowson (117) who, on a tetraploid *D. stramonium*, developed a scion of diploid *D. stramonium* in such a way that different branches grew above the same root. They did not contain the same quantity of alkaloid, however. On the contrary, the tetraploid branch was richer in alkaloid. The authors think that this is possibly due to an increased storage capacity in the tetraploid branch, although it does not preclude the possibility of differential rates of synthesis occurring in the branches. But we must also include the possibility that the migration of alkaloid from the root into the shoot might, in diploid branches, be more difficult across the graft. Finally, we must lay stress upon the fact that, without considering the well-investigated Solanaceae and lupines, only sporadic data exist about other alkaloid plants; e.g., the information of Moerloose & Ruyssen (163) reporting that *Cinchona* alkaloids are produced in the root and in the bark, but only stored in the leaves. Thus, it is not too presumptuous to think that the present opinion concerning the locus of alkaloid formation is too limited. Another complication lies in the fact that the alkaloids produced in the scion on alkaloid-free stock are not totally identical with those which the scion contains in its own root. But those alkaloids characterized by paper chromatography are not yet known exactly [Romeike (211)].

Translocation and Partition

A considerable amount of the alkaloids produced in the roots can be stored there. That is the case with berberine, of which adsorption at the cell walls renders the flow into other organs more difficult, as can be deduced from the investigations of Klein & Bartosch (137) with *Mahonia;* of Cromwell (33) with *B. Darwinii;* and of Greathouse & Watkins (77) on *Mahonia.* From the evidence of the few cases investigated, it may be deduced that the alkaloids, in most cases, flow upward with the general sap stream. The bleeding sap taken from carved roots or stems contains alkaloid in high concentrations, as already mentioned. Hieke [see Mothes (170)] succeeded in demonstrating this not only in *Nicotiana*, *Datura*, and *Atropa*, but also in *Berberis*, *Mahonia*, *Corydalis*, *Ricinus*, and *Veratrum*. With some caution we may conclude that, in these cases also, alkaloid is produced in the root. In young plants of *D. stramonium*, Hegnauer (88) finds a daily secretion of 0.35 mg. alkaloid in the bleeding sap and in older plants, which were intentionally prevented from flowering, he finds 2 to 3 mg. But he does not accept this as sufficient evidence to explain the quantity of alkaloid stored in the shoot. That, however, is not to be expected. Root pressure is not the only power which sets the sap stream in motion. What the transpiration-suction process is able to effect is unknown. Whereas Romeike (211) found only scopolamine in the bleeding sap of *Datura*, *Hyoscyamus*, and *Atropa*, Hegnauer (88) reports that very little scopolamine is to be found in *D. stramonium* but that hyoscyamine predominates. This is substantiated by Mothes *et al.* (185). They explain the primary differences by the assumption

that the sap flow, beginning shortly after the shoot has been cut off, perhaps originates from the contiguous parenchyma after damage and is not exactly bleeding sap. In tobacco, Schmid (218) found a continual decrease of daily nicotine secretion from the bleeding sap but did not find the variation of the nicotine decrease in agreement with that of the bleeding sap secretions. According to Schmid (218), nicotine forms the principal part of the organically bound nitrogen of the bleeding sap of tobacco. Evidently nicotine is the only product of the nitrogen assimilation of the root which flows into the above-ground parts. This strongly recalls our experiments with the bleeding sap of *Acer* [Mothes & Engelbrecht (176)] in which we found that allantoine and allantoic acid comprise the greatest part of the nitrogen in the bleeding sap, and of experiments with Betulaceae in which the same was true for citrulline [Wolffgang & Mothes (267); Reuter & Wolffgang (206)]. However, according to our investigations, the bleeding sap of tobacco also contains amino acids.

Hofstra (106) observed the root bleeding of *N. rustica* for several weeks. From one plant nicotine was secreted at a daily average of 17 mg. which was a considerable quantity. Compared with the quantity of nicotine contained in a root before the experiment, it follows that, within the period beginning September sixth to twenty-sixth, about 170 mg. of nicotine must have been newly formed without the help of a shoot. Also the experiments of Mothes *et al.* (185) on the bleeding of *Nicotiana* roots which received no nitrogen from outside but were nourished only by the leaves, support the theory of a continuous delivery of nicotine by the root.

Although the process by which the alkaloid enters the vascular system is still unknown, it may be supposed that the migration is passive, the alkaloid pulled along by the "bleeding" and transpiration stream. Also the fluorescence-optical test of nicotine in the xylem of tobacco [Schmid (218) favors this hypothesis.

Of course, this does not preclude movement in the phloem. In *Datura* scion on tomato, Hegnauer (87) microchemically discovered alkaloid in phloem and xylem. However, there are only a few positive and essential facts which serve to suggest an alkaloid migration downwards from the leaves into the basal parts. This is insufficient proof as Mothes (168) reported in 1928. In principle, the experiments of Hieke (99) and Mothes & Romeike (180) had the same result. One must not forget, however, that the same authors, grafting potato upon *Datura* root, found hyoscyamine in the tubers of the potato scion, but found 10 to 20 times more in the potato leaves. Hegnauer (90), too, reported alkaloids moving up and down. Of special interest are his graftings of *D. stramonium* upon tomato. He found mydriatic alkaloid in small quantities in all parts (flowers, young fruits, unripe seeds, ripe seeds, leaves, tomato stocks). An interpretation of these results is, according to Hegnauer, not yet possible, as it has not yet been ascertained whether the tomato species itself produces traces of mydriatic alkaloids. Romeike (211) demonstrated none in tomato plants. When graft-

ing *Atropa* on tomato she also found evidence of mydriatic alkaloid in the scion and the root.

Between the tomato root and tomato shoot Laschuk (148) grafted a segment of tobacco root which produced alkaloid if it includes the root tip. This alkaloid moves not only into the scion but also into the root.

The earlier works of Kuzmenko & Tikhvinskaja (147) indicate that an alkaloid transport takes place from the scion into the stock. They grafted *N. glauca*, which produces anabasine in the leaves, on *N. tabacum* and *N. rustica* and found anabasine also in the stock which, ungrafted, contains no anabasine. But in our experience, it is rare to find *Nicotiana* free of anabasine. In any case, in their experiments (147), the influence of the stock upon the scion was stronger than that of the scion upon the stock.

Warren Wilson (266) reported a number of convincing experiments. For example, in grafting *Atropa* on tomato, the scion, experimentally induced to develop roots by itself, was producing species-specific tropane alkaloids, a phenomenon which, however, could also be demonstrated in species-foreign stock. She indicated alkaloid migration in the phloem, also, but suggested that the leakage of alkaloids from xylem to phloem seems very probable. Such a leakage of other substances from xylem to phloem is also discussed by Kurssanov & Saprometov (145).

The mechanism of substance migration, however, is insufficient to be solely responsible for the distribution of alkaloid in the shoot. For some time it was commonly believed that transpiration was the decisive factor in distribution because, following transpiration the alkaloid remained. However, this concept corresponds, in general outline only, to the existence of alkaloids in the principal organs of transpiration, the leaves. More exact investigations suggest another view. Hegnauer (88) shows that the accumulation of alkaloid in a tomato scion upon *Datura* is different from that found in a normal *Datura* plant. In the case of the tomato scion, the lamina of the leaf has the highest percentage of alkaloids, whereas the flowers and young fruits contain very little. The distribution could be accounted for as being a purely passive accumulation. However, in normal *Datura* and *Atropa* plants, the alkaloid distribution is such that it cannot be explained as the result of purely passive transport and accumulation by the transpiration current flowing through the plant. Hegnauer (90, 91) hints at a different method of distribution. In *Datura*, *Atropa*, and *Hyoscyamus* the highest concentration is found in the petiole and in the midrib, but in tomato upon *Datura*, it is in the blade. Olsson (190), Romeike (211), and Mothes & Romeike (182) came to similar conclusions. In leaves of *D. innoxia* the mg. of alkaloid per 100 gm. of fresh weight (mg. per cent) in blade, veins, and petiole have a ratio of 18:120:55; in *Cyphomandra* upon *D. innoxia*, however, the ratio is 45:15 (−22):9; in *Atropa*, 30:80:55; in *Cyphomandra* upon *Atropa*, 50:25 (midrib); in tomato upon *Atropa*, 100:50 (rachis). If *Atropa* is grafted on *N. rustica*, the nicotine distribution in the *Atropa* leaf is a complete reversal of the hyoscyamine distribution in the normal *Atropa* leaf. The

blade contains 45 mg. per cent, and the veins 6 mg. per cent of hyoscyamine (fresh weight); the outermost parts of the blade contain 130 mg. per cent and the inner part near the midrib only 2 mg. per cent of nicotine. Romeike (211) found that in *Atropa* upon *Nicotiana* the nicotine migrated into the scion, and was found especially in the blade, but the scopolamine was produced in the veins of the scion itself.

From the evidence, it must be concluded that, in addition to an alkaloid synthesis in the leaf itself, some mechanism of accumulation must play a decisive role as explanation for the species-specific alkaloid hyoscyamine and the species-foreign nicotine. This conclusion is sustained by the fact that, after artificial injection of scopolamine, hyoscyamine, strychnine, and chinine into *Datura* scions upon tomato roots, higher alkaloid concentrations were noted in the midribs [Hegnauer (90)]. A different reaction of leaves towards different alkaloids is further indicated by the fact that, if alkaloid-free leaves of *Atropa*, developed upon tomato root, are placed in a solution containing both hyoscyamine and nicotine, both are drawn in by the transpiration current. However, if the leaves are later placed in water, the nicotine returns to the water while the hyoscyamine remains in the leaf [Mothes & Romeike (182)].

A half-quantitative test of nicotine injected into *Atropa* leaf by grafting or spraying, may be made by the chloroplast-bleaching method which demonstrates considerable differences in the nicotine distribution. For example, the chloroplasts of the stomata and those of the parenchyma cells attending the veins show fluorescent chloroplasts for a long interval, whereas all others are bleached out.

Hofstra (106), too, contributes to this problem. He finds that in *N. rustica* the nicotine content expressed on a dry weight basis is approximately equal in young or old leaves and parts of the stem. But on a fresh weight basis, the higher leaves and the xylem were found to contain more than the pith and the bark. The marked variation in the proportion of nicotine to nornicotine is remarkable. In the leaves it is 1 to 2, in the axes 1 to 8, and in the xylem 1 to 20. This leads to another question: to what extent does the distribution of different alkaloids depend upon secondary variations of the alkaloid mixture in the scion?

How complicated the circumstances in grafts can become, is shown by the alkaloid content of alkaloid plant parasites. Walzel (258) reports that *Cuscuta gronovii*, parasitic on *Nicotiana*, remains alkaloid-free although the tracheids of the parasite enter the xylem of the host. Similar facts were reported by Mothes (172) for *Orobanche* species, parasitic on *Nicotiana* roots. They, too, remain free of alkaloid. This result must be investigated still further for there is not sufficient evidence to support either an assumption of a capacity of the *Orobanche* to split up nicotine, or of a physiological bar between host and parasite for which, at the present time, the anatomical postulates are unknown.

Alkaloids in Fruits and Seeds

If tomato is grafted on *Nicotiana*, *Atropa*, *Hyoscyamus* or *Datura* root and fruits are developed on the scion, no alkaloid will be found in them according to certain investigators [Vincent & Dulucq-Mathou (253); Warren Wilson (266)]. According to others, however, small quantities will be observed, but less than in the leaves of the scion [Javillier (126); Kerkis & Pigulevskaja (131); Krajevoj & Nechaev (141) Lowman & Kelly (153); Hegnauer (88); Mothes & Romeike (180); Romeike (211)]. At present, there is no doubt about the truth of this latter opinion.

This fact seems the more remarkable since the fruits of the alkaloid plants themselves, in most cases, contain alkaloid oftentimes a great deal more than do other organs. Thus, here too, it is not possible to imagine an undirected distribution by the transpiration current. In no sense do tomato fruits present a special case. The same may be said for *Physalis*, and, most particularly for *Datura* on *Nicotiana:* the *Datura* fruits have no nicotine (Vincent & Dulucq-Mathou).

Mothes & Romeike (180) devised experiments to determine whether the small quantity of alkaloid of such tomato fruits might not result from destruction of translocated alkaloid. They injected nicotine and atropine into unripe and ripe fruits, but could not observe any significant degradation. Conversely, the question was examined whether the fruits of alkaloid plants synthesize their own alkaloid. In half-ripe detached fruits of *Nicotiana* there was never found, after subsequent ripening, an increase of alkaloid. On the contrary, half-ripe, detached seeds of *Datura* showed a clear alkaloid increase, which may have been the result of the activity of the radicle of the embryo.

Since the separation of the seeds from the placenta entirely changes the conditions of growth, the natural distribution of alkaloids in seeds and fruits cannot be deduced from these experiments.

More important are the alkaloid analyses of fruits and seeds of alkaloid-plant scions upon alkaloid-free stock. Kerkis & Pigulevskaja (131) found, upon grafting *D. stramonium* to tomato, that there were only insignificant quantities of atropine, if any, in the fruits of the scion.

Other authors, [Mothes & Romeike (180)], report larger quantities of alkaloid, e.g., in *D. stramonium* fruits upon tomato roots, but state that these alkaloids have little mydriatic effect. Therefore, they cannot be hyoscyamine exclusively. Similar difficulties in the diagnosis of alkaloids arose in *Nicotiana* fruits on tomato root; only part of the alkaloid observed was nicotine. In this case, however, the question whether this alkaloid is produced in the shoot cannot be answered easily, since the tomato root also produces nicotine in small quantities.

Therefore, there cannot be any doubt about the considerable difference between the alkaloid content of fruits upon species-specific root and those upon species-foreign root. Although one cannot conclude, that alkaloid in

the fruits, as well as the leaves, depends on the native root, it is just as impossible to conclude that the high content found in fruits developed upon their own root is synthesis *in situ*. The situation can, perhaps, be described in the following manner: the specific root produces certain substances which favour alkaloid synthesis in the fruits.

In this connection, we may refer to the investigations of von Sengbusch (230) and of Hagberg (83). In crosses of "sweet" and "bitter" lupines, they find evidence of an important maternal influence on the alkaloidal characteristics of the seeds. Plants from seeds on a "sweet" mother do not show their genetic "bitterness" until rather late in their ontogeny. Genetically "sweet" plants, grown on "bitter" plants, do not show the "sweet" tendency until rather late. This indicates a translocation into the seeds rather than synthesis *in situ*.

The intricacy of the situation is evident in the reaction of different tobacco species. Iljin (110, 111, 112) has shown that half-ripe seeds of *N. tabacum* contain nicotine in considerable quantities which disappears during increasing maturity. But Schmid & Serrano (219) and Mothes & Romeike (180) proved that nicotine is found regularly in ripe seeds of *N. rustica*. In the phases of development in which nicotine decreases in tobacco seeds, the alkaloid of the *N. rustica* seeds increases; nornicotine increasing to a higher degree than nicotine.

A final answer to the question of how completely the alkaloids in seeds and fruits may be explained by synthesis *in situ*, degradation, translocation, and migration will be best achieved by employing alkaloids which are radioactively marked.

Secondary Chemical Transformations of the Alkaloids

According to Dawson (41, 42, 43) tomato scion upon *N. glauca* contains anabasine and nicotine; *N. glauca* upon *N. tabacum* contains anabasine and nornicotine, and upon tomato, anabasine alone. He concluded that anabasine and nicotine are formed in the root of *N. glauca*. Moreover, anabasine is formed without assistance and independently of a *Nicotiana* stock in the leaf. Žukov (269) and Iljin (113, 115) are of the opinion that nicotine, translocated from the stock, may be transformed into anabasine. This suggestion requires substantiation.

Transformation of nicotine into nornicotine, however, has been proved by Dawson (42, 43) and by Iljin (114a). Certain strains of *N. glutinosa*, *N. silvestris*, and *N. glauca* are capable of this transformation, as are *N. tabacum* and *N. rustica*. In this case, it is a matter of a demethylation limited to intact leaves [Iljin (115, 116)] and probably proceeding by the common transmethylation mechanism. Nicotine infiltrated into detached leaves may be transformed into nornicotine [Iljin (113)]. In certain *Nicotiana* species this demethylation seems to become important in older plants only. Later on

it can cause nicotine to disappear entirely. Apparently, such a process also continues during the time of drying, although quite different interactions then interfere [Wahl (255, 256)].

Certainly, it is not justifible to ascribe solely to the leaf the capacity of demethylation [Dawson (42, 43)]. We agree with Hofstra (106) that nornicotine can also exist in the root. Whether it is there as a precursor or a product of nicotine is not quite clear. But according to Hofstra, the proportion of nicotine to nornicotine is smallest in the leaf.

Much discussion now centers on the transformation of the tropane alkaloid. Evans & Partridge (59, 60) made grafts involving *D. tatula*, *D. ferox* and *D. innoxia*. *D. tatula* normally contains 0.12 per cent scopolamine and 0.24 per cent hyoscyamine; *D. ferox* 0.31 per cent scopolamine, 0.10 per cent meteloidine and 0.11 per cent of other alkaloids. In the scions of reciprocal grafts the proportion of the alkaloids was the same as if the scion were grown upon a root of its own. Thus, *D. ferox* upon *D. tatula* contained scopolamine and meteloidine and *D. tatula* upon *D. ferox*, scopolamine and hyoscyamine. The authors conclude that *D. tatula* synthesizes hyoscyamine, and *D. ferox*, meteloidine in the shoots. It is postulated that the roots of both *D. ferox* and *D. tatula* are either incapable of synthesizing meteloidine and hyoscyamine or that translocation of these alkaloids from the stock to the scion does not occur. The grafts of *D. ferox* and *D. innoxia* bear out this concept or, at least, do not refute it. The assumption is that the last steps of alkaloid synthesis are accomplished in the shoot.

James & Thewlis (125) learned, however, that in grafts of *A. belladonna* and *D. innoxia* the alkaloid character of the scion was dependent on the respective root. But Evans & Partridge (60, 61) have expressed doubt about this observation.

Hegnauer (91) and Romeike (211) found no indications that scopolamine may be transformed into hyoscyamine in the leaves of *Datura* species. Alkaloids experimentally applied to the shoot remained constant. There is no doubt, according to the investigations of Cromwell (35), Hegnauer (88 to 91) and those of Mothes *et al.* (185), that bleeding sap of different mydriatic Solanaceae contains hyoscyamine. Hills, Trautner & Rodwell (105) grafted *N. tabacum* upon *Duboisia myoporoides* and found, in the scion, nicotine, nornicotine, scopolamine, hyoscyamine, and tropine. They explain the existence of tropine by the assumption that the free base is produced only in the root; the ester later on in the leaf. It is possible, however, that the esters are hydrolized in the leaf.

An argument in favour of a transformation of the alkaloids may be observed in *Smirnovia turkestana* in which is formed smirnovine in May, but which produces smirnovinine and sphaerophysine in August [Rjabinin & Iljina (207)]. Similar development-dependent changes of the proportion of alkaloids are also known to take place in *Duboisia*. A true transformation, however, from one type into another has not been proved.

Degradation and Excretion of Alkaloids

Hitherto, very little has been known concerning physiological degradation of alkaloids. The loss of alkaloids in organs of the plant are never convincing, since translocations may also be involved. A slight nicotine decrease in detached old leaves of tobacco was observed by Mothes (168). In similar experiments, Gorter (75) sustained leakages. Vickery & Meiss (251) observed only small nicotine losses during the curing of tobacco leaves. Frankenburg (65) and Nisoli (188) had similar results. The latter reported, moreover, that the alkaloid content increases when intact plants fade in the open air. The reason for this phenomenon seems to be a translocation from stems and roots. Hegnauer (91) placed detached scions of *D. stramonium*, grown upon tomato, into solutions of hyoscyamine and scopolamine. He did not find a degree of degradation sufficient to warrant comment. Similar results were reported by Romeike (211).

On the other hand, there are a great many statements in the literature which point to a probable decrease, during maturity and senescence, of the total alkaloid within each plant. There is the question, too, of whether the quite noticeable alkaloid decrease in attached old leaves should be attributed, in part, to a translocation into younger parts. James (119), reports that the alkaloid content of matured *Atropa* leaves decreases noticeably, not only relatively (in per cent dry weight) but also absolutely: 1.37 mg. to 0.32 mg. (in one leaf).

Isolated unripe fruits of *N. rustica*, having ripened for eight days after being gathered, show a considerable loss of alkaloid in the pericarps although we were unable to detect a major movement into the seeds [Mothes & Romeike (180)]. In this connection there must again be reference to the physiological nicotine loss in ripening tobacco seeds, a process which finally produces an alkaloid-free condition [Iljin (110, 111)]. In *Atropa* scions upon *N. rustica* Mothes & Romeike (182), too, observed a large nicotine loss during the necrotic drying of leaves injured by nicotine. From these results must be eliminated all of those in which the influence of bacteria must be considered [Wenusch (263)]. Such investigations as well as the effect of animal tissue upon alkaloids, cannot be discussed here.

For the establishment of alkaloid balances the excretion of alkaloids through the cuticle is highly important [Chazé (28 to 31); Mothes (169); Schratz & Spaning (228)]. Undoubtedly, this problem is important, too, in the cultivation of alkaloid plants, for frequent moistening by rain can wash away 50 per cent of the alkaloids accumulated in the leaves [Mothes (170)], although the amount of loss depends upon the type of alkaloid (nicotine washes away easily). Unquestionably, this is one of the causes of the small alkaloid content found in leaves in humid seasons [Hofstra & Keuls (107)]. As for nicotine, the possibility of gaseous excretion must also be considered.

Not yet investigated on a physiological basis is the excretion of alkaloids into the soil from the roots of intact plants. This process is of great ecological importance, for such elements may decisively influence microorganisms as well as higher plants.

Influence of the Scion on Alkaloid Production by the Root

Since alkaloid production must, in some way, depend upon the activity of root cells, all effects exerted by the shoot and therefore by the scion upon this root activity, may influence alkaloid production as well.

Indeed, not only do the alkaloid concentrations in different scions upon the same stock vary greatly, but so do the absolute quantities of alkaloid transmitted by a root to the scion. Thus, in grafts of *D. innoxia* upon *N. rustica*, the percentage of nicotine obtained is far greater than in *N. rustica* itself [Mothes & Romeike (182)]. The growth of the scion here is greatly inhibited. This is not so in grafts of *C. betacea* upon *D. innoxia* in which the leaves of the scion may contain up to six times the quantity of scopolamine found in the leaves of the *Datura* controls [Mothes (172); Mothes & Romeike (182)]. The total quantity of alkaloids in such a *Cyphomandra* scion is many times that which is produced by a normal *Datura* plant. Moreover, this graft survives for several years, the leaves growing older than those of *Datura*. The question of the possible amount of alkaloid production must, of course, be investigated in relation to the root itself, its growth, and its proportion of active young tissue. To date, no inquiries have been made in this direction.

Of special interest are the grafts of a chlorophyll-defective, tomato mutant "Xantha" upon *Nicotiana* root. These scions contain less than one-tenth of the amount of nicotine found within the tobacco shoots themselves, or within the green tomato scions upon a similar *Nicotiana* root. The cause of this remarkable fact is in doubt. It may be suppression of production in the root by the nonassimilating scion or a degradation of nicotine in the scion itself.

In 1941 Schmuck, Smirnov & Iljin (223) reported that a scion can contain a species-foreign alkaloid in higher quantities than that contained in the stock, e.g., *Solanum nigrum* upon *N. tabacum*. Laschuk (148), too, lays stress upon the reciprocal influence of the organs during the formation of alkaloids in the root. Alkaloids are products of the metabolism of the whole organism.

Of special interest is the announcement made by Jackson & Rowson (117) that tetraploid tomato scions cause greater alkaloid production in the tetraploid *D. stramonium* stock than do diploid tomato scions. Certainly, in this activity of the scion not only are general food influences decisive but hormonal relations are important as well. However, until the root itself is measured and analyzed within the framework of these experiments, the relationships will remain unclear.

The Ontogenesis of Alkaloids

In 1950, Guillon (80), reporting on the basis of microchemical tests of *D. stramonium*, stated that not until the sixth day of germination, when the cotyledons turned green, did alkaloids appear in the peripheral tissues of the root, continuing to spread thereafter to the endodermis. In the course of

one month, they reached the pith, the cork, and the phelloderm. During fading, the roots again lost alkaloid, with only the central cylinder giving a positive test. In the stem alkaloid appeared at about the tenth day of germination, first in the parenchyma of the bark, later in cork and pith. In the leaves it was first found in the epidermis, with only traces in the corolla. Such loss of alkaloid in the roots is especially distinct for hordenine in barley seedling [Raoul (201)].

It has been repeatedly determined that the youngest leaves are especially rich in alkaloid on a dry or fresh weight basis [Mothes (168); Meyer (162); James (119); Hegnauer (87); Olsson (190)]. Perhaps during later development, although the leaves increase in absolute alkaloid content to a maximum, the content as a percentage of weight continuously decreases. This is not true with grafts. In *Cyphomandra* upon *Datura* or *Atropa*, as in *Atropa* upon *Nicotiana*, and in *Lycopersicon* upon *Atropa*, the youngest leaves contain considerably less alkaloid than the older ones [Romeike (211); Mothes & Romeike (182)]. If one does not wish to accept this as proof of alkaloid synthesis in the youngest parts of the shoot on the species-specific root, it might more readily be assumed that the translocation and partition of specific and foreign root alkaloids are different. Still more striking are the important variations in the mixtures of alkaloids in the course of growth in different parts of the plant. Nisoli (188) states that for *D. innoxia* the proportion of hyoscyamine to scopolamine is much greater in the roots than in the stem, and greater in the stem than in the leaves.

James & Thewlis (125) report, on the contrary, that the proportion of alkaloids in the different organs of the same plant most likely changes in the course of development, and in all parts, each change is both identically and simultaneously made.

Romeike (211) could substantiate this only in part. In partial agreement, also were Hegnauer (89), Evans & Partridge (61), and Jentzsch (128). Romeike found, in most of the mydriatic Solanaceae mainly scopolamine in the young plants, hyoscyamine in the old [cp. Trautner, (246)]. However, for this condition, there are certain exceptions. More important appear to be the results of Romeike. She found, in several species of Solanaceae, primarily in the young root, evidence of not yet definable alkaloids with fixed Rf values, which may be precursors of the tropines. Van Haga (82), too, reports similar results, stating that in the germination of *A. belladonna* there appears as the first base a not yet identified substance, probably bellaradine. A notation of Hegnauer (94) suggests that it is probably cuskohygrine. In young plants and also in fruits, moreover, tropine and scopine or scopoline can be demonstrated. For the present it is a question of whether they are precursors or decomposition products of ester alkaloids [Romeike, (211)].

In numerous cases the development of alkaloid content reaches a maximum during, or a short time before, flowering [cp. Ahmed & Fahmy (2) with *H. muticus;* Blažek & Kučera (10) with mydriatic Solanaceae; Mitsuchashi & Imazeki (162a) with *Scopolia japonica;* Schpilenja (227) with *H. niger*

and *Atropa;* Rudorf & Schwarze (213) with *Datura;* Nisoli (188) with *Datura*].

Unfortunately, only in a few cases is the absolute content of alkaloid in a whole plant reported, e.g., Haller (84) with *D. innoxia.* Within seven weeks the alkaloid content drops to a fifth; a loss which is impossible to interpret as a leaching out or as a falling of old leaves. On the contrary, considerable quantities of alkaloid seem to be destroyed or transformed.

Indeed, the formation of alkaloids in the root seems to diminish from the moment of flowering. Unfortunately, in these experiments the growth of the root itself was not measured exactly enough. One may suppose that hormonal influences proceed from the flowers inducing the general activity of the root. Thus, Mothes & Romeike (182) found, in grafting *Atropa* upon *N. rustica,* that much less alkaloid existed in graft and root at the beginning of flowering than before, and that cutting short the development of the scion caused a quick increase of alkaloid in the root and in the temporarily regenerated vegetative scion.

This phenomenon serves as a reminder of the well-known fact that cutting of the terminal and lateral shoots as well as the flower buds, causes a considerable increase of the alkaloid content in the remaining parts of the plant [Schmuck (221); Hegnauer & Flück (95); Prasad (197); Nisoli (188); Laschuk (148); Bynow (25); Hofstra & Keuls (107)]. It cannot be a question of retaining these alkaloids which, in normal plants, would have been moved into flowers and fruits [Schmid (218); Prasad (197)], but is a matter of an increased production [Nisoli (188); Bynow (25); Hofstra & Keuls (107)]. Laschuk (148), and Hegnauer & Flück (95) also state that increased vegetative growth increases the metabolic activity of the root. In this respect, the influence of flowering on nitrogen absorption and protein metabolism of *Cannabis sativa* should be mentioned [Mothes & Engelbrecht (175)]. In male plants nitrogen absorption completely stops when the flowering begins. At the same time, proteolysis begins and considerable excretion of soluble nitrogen into the nutrient solution may take place [Mothes (172)].

It would be of great importance to know whether the decrease in alkaloid content of aging alkaloid plants is likewise associated with excretion by the roots. By such a study numerous uncertainties in the present publications would be eliminated.

We must not overlook the fact that not all of the authors observe the suspending effect of flowering upon the alkaloid content [Schmid & Serrano (219); Vladescu (254)]. Since, obviously, the phenomenon of flowering is hormonally controlled, it was logical to search for a hormonal regulation of alkaloid formation in the root. But Hegnauer & Flück (95) found no significant effect when they sprayed with 2,4-dichlorophenoxyacetic acid. Tsao & Youngken (247) obtained similar results and believe that there is no close relation between growth and alkaloid formation.

Schmid & Serrano, in part because of results obtained by Vladescu, made a comparison of the total alkaloid production of the root and its al-

bumen content. The curves were inclined to be parallel. And in the culture of excised root tips of *Lupinus*, Mothes & Kretschmer (179) found the alkaloid content so nearly proportional to the dry weight of the root, it was thought that an immediate connection probably exists between alkaloid synthesis, albumen production, and growth of the root. However, this assumption requires thorough investigation. The fact that roots, separated from the shoot, are able to produce alkaloids for a period of weeks [Hofstra (106); Mothes *et al.* (185)] rather contradicts this conclusion. This question must be studied again with careful observation of the growth of the root.

If, therefore, the alkaloid content, during the course of a year, fluctuates with the changing growth pattern we may raise the question whether this phenomenon obtains during the course of a day. Mothes (167) has already shown the difficulty of answering this question because of the high variability of the material in relation to which the alkaloid concentration is expressed. Hemberg & Flück (96) found a higher alkaloid content in *D. stramonium* in the morning. Future investigations will reveal whether synthesis or translocation occurred during the night.

External Influences and Alkaloid Synthesis

Nutritional conditions greatly influence the alkaloid concentration and total alkaloid content in any one plant. A great number of investigations have been made [e.g., James (120); Schmuck (221); Gstirner (78); Nisoli (188); Gilmore (72); Hofstra & Keuls (107), etc.]. But the results obtained are at variance with each other. In some cases, potassium is asserted to have a positive effect on alkaloid production; in others, the contrary is reported, e.g., Shibata & Imaseki (231). Moreover the origin of the nitrogen influences the alkaloid content. However essential the practical importance of these investigations may be, for the present, they contribute little to an increased knowledge of the biosynthesis of the alkaloids, since the root system is not yet sufficiently understood. Moreover, the determination of whether the physiological condition has an indirect effect upon growth or a direct one upon the alkaloid formation—remains problematic. This whole complex of questions, development, growth, and alkaloids requires more exact studies. Light is not necessary for alkaloid production as long as glucose is available [Cromwell (34)]. The experiments with detached leaves referred to above have established this point. For the root good oxygen supply is essential [Haller (84); Ahmed & Fahmy (2)]. Thus, in damp soils alkaloid production appears to be considerably less [Bavel (7)].

To comprehend the total complex of climate seems much more difficult. The statistical investigations of Renier (205) indicate that with tobacco the years of highest insolation produce leaves richest in nicotine, whereas the relation of nicotine content to dryness is not so evident. This is remarkable as one might suppose that a higher temperature in the leaf would increase the excretion and evaporation of nicotine. The statements of Hills & Rodwell (101) regarding clones of *D. myoporoides* indicate that climatic factors exert

a strong influence upon alkaloid content. The composition of the alkaloid mixture which changes from year to year may also be attributed to climatic causes.

According to Hegnauer (93), the climate has no influence upon alkaloid content in the young root tubercles of *Aconitum napellus*. The same is true for the alkaloid content of *Cinchona* bark. On the other hand, according to Hegnauer, the alkaloid content of plants with an intensive cuticular excretion of alkaloids is sensitive to climatic conditions, especially rain.

Haud, Kapoor & Chopra (86) found that in *H. niger* cultivation at greater altitudes (5000 ft.) increases the alkaloid content. It is apparent that the relation between climate and alkaloid synthesis have been less well-investigated than that involving nutritional conditions.

The Mechanisms of Biosynthesis of Alkaloids

The high merit of the work done by Robinson and Schöpf in the development of chemical theories on "biological" conditions is well-known. Summaries of their recent results are given by Pailer (192), and Hughes & Ritchie (109). Mention should be made also of Schöpf's work in 1952 (224) concerning morphine synthesis. It is doubtful whether these interesting experiments really are patterns of natural processes. So much evidence favours the supposition that nature prefers quite different methods. Too, it is not clear whether the experiments on the mechanisms of biological degradation permit conclusions about biosynthesis.

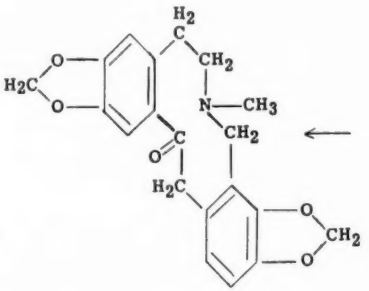
It is evident that the biosynthesis consists of a large number of reaction steps. One such reaction which can be adequately explained by knowledge acquired within the last years, is the incorporation of methyl groups, especially at the nitrogen atom.

This methylation occurs according to a mechanism of transmethylation discovered by Du Vigneaud, in which methionine first acts in the capacity of a methyl group donator and itself is changed into homocysteine. In the same manner, guanidino acetic acid can, in animal tissue, be transformed into creatine. But an activation of methionine is needed in which ATP is required. In the first instance, a S-adenosylmethionine is likely to be produced which represents the true methylating agent [Cantoni (26)]. Other CH_3 donators, choline, betaine, and demethylpropiothetine (isolated from the alga *Polysiphonia fastigiata*) etc., were discovered later. The mechanism first observed in the animal organism has its counterpart in plants [see Barrenscheen & Vályi-Nagy (6); Steensholt (237)]. By such methylation, sarcosine can be produced from glycine, choline from amino ethanol, and even methionine [see Challenger (27)]. Later it was known that this transmethylation is a reversible reaction; methionine not only participates in choline synthesis but choline takes part in methionine synthesis. It is possible that choline is first oxidized to betaine.

By this transmethylation mechanism, trigonelline combines with niacin. Zeijlemaker (268) demonstrates that, in green pea plants niacin increases

absolutely in the whole plant up to the seventh week and then decreases. In the leaves, as in the root and stem, the absolute content drops with the beginning of flowering. If the cut shoots receive niacin artificially, the niacin content of the shoot remains nearly constant. The total excess niacin is transformed into trigonelline. This synthesis is not promoted by tryptophan, ornithine, citrulline, pyridoxine, nor pyruvic acid.

Such methylation at the nitrogen atom is observed in the synthesis of methyltyramine and hordenine [Kirkwood & Marion (134, 135)]; of nicotine [Brown & Byerrum (21)]; and of ricinine [Dubeck & Kirkwood (53)]. In this last alkaloid the same transmethylation mechanism is evidently as effective as in the synthesis of the methoxyl group. This important result is confirmed by the fact that the oxygen methyl groups of barley lignin are formed in the same pattern [Byerrum & Flokstra (23)]. Sribney & Kirkwood (234) found that not only the nitrogen methyl group of protopine, but also the two methylendioxy groups come from the labile CH_3 groups of methionine. Formate has very little activity, and it is possible that this "single carbon" precursor enters into the place marked in the protopine molecule depicted below.



Protopine

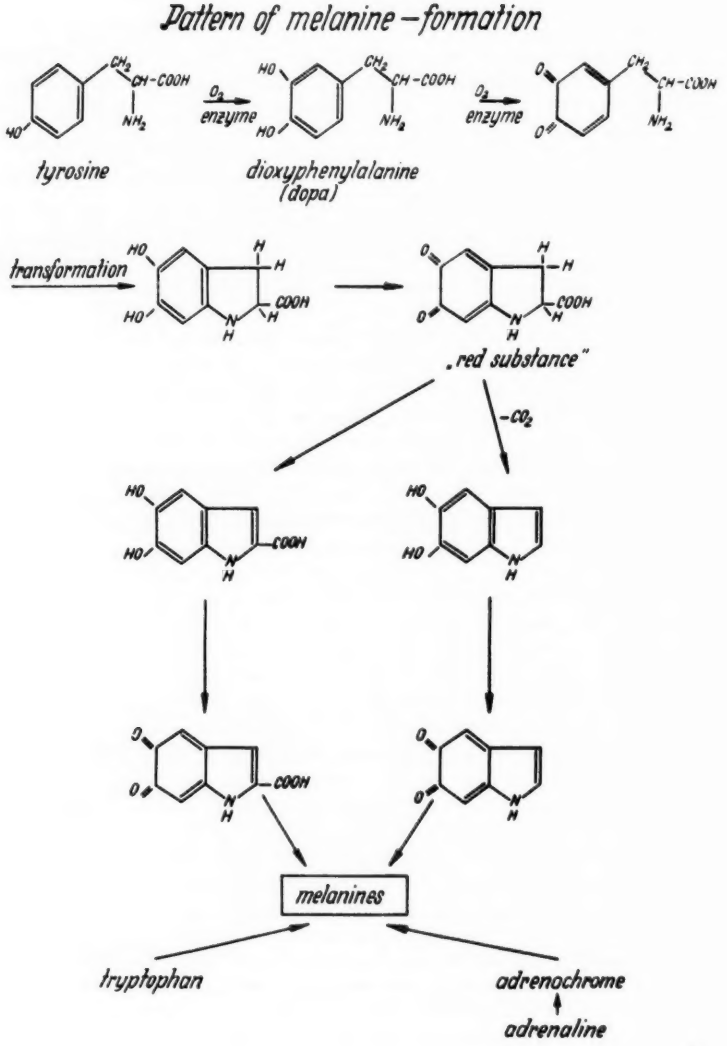
In this case choline is as inactive as in the biosynthesis of ricinine (see above) and hordenine [Matchett, Marion & Kirkwood (159)]. In all these experiments the use of C^{14}-labelled methyl groups was responsible for the decisive success. According to Byerrum & Wing (24), in the biosynthesis of nicotine the methyl groups of choline are transferred as rapidly as those of methionine. During the feeding of tobacco with C^{14}-labelled choline, no choline could be found in phospholipides. Brown & Byerrum (21) transplanted *N. rustica* var. *humilis*, a high nicotine strain grown in soil, to tap water which contained inorganic nutrients as well as methionine which was labelled with C^{14} in the methyl group, and formate. The nicotine formed in the root of the tobacco contained a labelled methyl group. Methionine had higher activity than the formate. This result suggested that formate

may be used by the plant in the synthesis of labile methyl groups which then undergo transmethylation to nicotine.

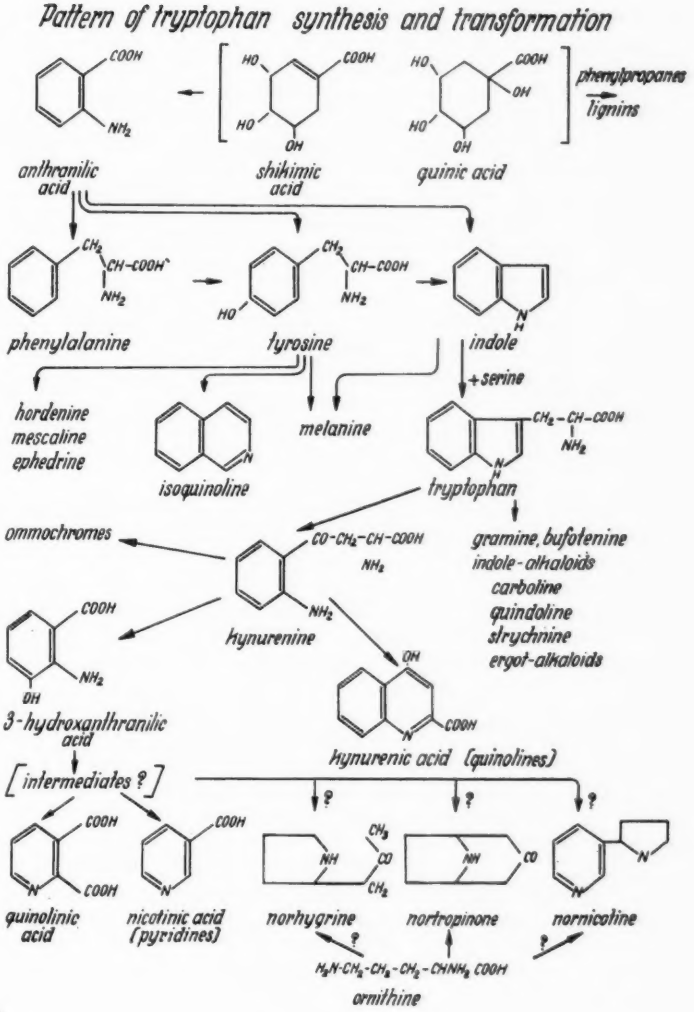
Kuzin & Merenova (146) darkened attached tobacco leaves for 48 hr., cut them off, and placed them under artificial light for 24 hr. in an atmosphere of $C^{14}O_2$. The nicotine isolated contained C^{14} in the methyl group. From this result it must be concluded that the methyl group of the nicotine is labile and can be exchanged.

There is no doubt that the central problem of alkaloid biosynthesis lies in the formation of heterocyclic rings. A number of important results in this field are under discussion which, strictly speaking are reported from outside the province of proper alkaloid research. First must be noted the biosynthesis of melanin [Raper, Danneel, etc.; for summary cp. Karlson (129)]. This process is an excellent example of the influence of genes upon the development of a chemical characteristic. There seems no longer to be any doubt that the same method of development is followed in the formation of colours by mammalia, insects, higher plants, and fungi, although special differences in detail apparently exist as to the material (see Pattern of melanine-formation, I), the intermediates, the degree of polymerisation, and the insignificant chemical variations of the melanin. We must not forget that most of the reports have their origin in experiments using enzymes *in vitro* and that *in vivo* many more variants may be possible than those already suggested by the great gradation of colours from yellow to black.

Mention should be made of the investigations concerning the synthesis of the ommochromes from tryptophan (see Pattern of tryptophan synthesis and transformation, II) (Kühn and Butenandt, Beadle and Ephrussi, and Tatum); of the relations between tryptophan and nicotinic acid; and of the formation of the indole ring itself [Tatum & Bonner (243); Umbreit, Wood & Gunsalius (250)]. These investigations involve different circles of collaborators [(cp. Butenandt & Renner (22); Stanier & Hayaishi (236); Karlson (129); Sanadi & Greenberg (215); Galston (70); Beadle (8); Nason (186); Nyc & Mitchell (189); Partridge, Bonner & Yanofsky (195)] and relate to animals, fungi (*Neurospora*), and higher plants. They have made clear that from tryptophan pathways lead to the pyridine (nicotinic acid) and to chinoline compounds (kynurenic acid). Since, on the one hand, tryptophan results from tyrosine and since tyrosine not only leads to the tyramine derivates (hordenine, etc.) but also to the isochinolines, and, on the other hand tryptophan is related to gramine and to all the other indole alkaloids, the system tyrosine-tryptophan becomes the center of a great many plant bases. It is of fundamental importance to know that substances of such different character as isochinoline, chinoline, pyridine, and indole are biochemically related to each other. Since tyrosine itself is closely related to the phenylpropane substances and shikimic acid and quinic acid, here, too, result natural relationships between the heterocyclic compounds and the aromatic ones. It is not clear whether the hygrines, the tropinones and the nicotines can also be included in these associations [Mortimer (165)].


Pattern of melanine-formation
tyrosine
dioxyphenylalanine (dopa)
transformation
„red substance"
melanines
tryptophan
adrenochrome
adrenaline

I


Pattern of tryptophan synthesis and transformation
anthranilic acid
shikimic acid
quinic acid
phenylpropanes
lignins
phenylalanine
tyrosine
indole
+serine
hordenine
mescaline
ephedrine
isoquinoline
melanine
tryptophan
ommochromes
kynurenine
gramine, bufotenine
indole-alkaloids
carboline
quindoline
strychnine
ergot-alkaloids
3-hydroxanthranilic acid
kynurenic acid (quinolines)
[intermediates ?]
quinolinic acid
nicotinic acid (pyridines)
norhygrine
nortropinone
nornicotine
ornithine

Feeding experiments with β-labelled DL-tryptophan have shown that the roots of young tobacco plants do not produce radioactive nicotine. This, however, does not mitigate against the suggested relations [Bowden (14)].

However significant this knowledge of tryptophan metabolism may be, it cannot be contended that the alkaloid ring systems are synthesized by the same pathway. But, for the first time, we possess several established, essential facts with which to explain the ability of the organism to produce such systems, how it opens rings and closes them again, thereby incorporating new chemical groups.

Compared with these successes, the experimental facts won in reference to alkaloids are very moderate. After Raoul (201–203) found the relationship between tyrosine and hordenine in the young roots of germinating barley, Leete, Kirkwood & Marion (150) demonstrated that tyramine, labelled at the α-C atom with C^{14} and administered to sprouting barley, leads to the synthesis of hordenine and N-methyltyramine which, at the α-C atom, are radioactive. The activity of methyltyramine is ten times greater than that of the hordenine. DL-Tyrosine-2-C^{14} administered to sprouting barley also produces radioactive hordenine and methyltyramine. The activity is to be found in the second carbon atom of the side chain [Leete & Marion (151)]. Kirkwood & Marion (134) and Erspamer & Falconieri (57) report similar results.

Whereas hordenine has its origin in the root, gramine seems to be produced in the tips of the barley leaves. At any rate, when barley is fed tryptophan with C^{14} in the β-carbon, labelled gramine is first observed there.

As for the much-discussed nicotine, Klein & Linser (138) reported in 1933 that, during a nutritional investigation of a decapitated tobacco plant using 1 per cent solutions of proline, glutamic acid, and ornithine, a considerable increase in nicotine takes place. Gorter (75) did not confirm these results.

Dawson (37) reported in 1939 large increases in nicotine in tobacco leaf blades when the excised shoots were fed nicotinic acid. In 1948, he repeated these experiments with nicotine-free tobacco leaves and failed to obtain a synthesis from proline and nicotinic acid. In experiments with detached tobacco leaves fed with amino acids, Schmid (218) could not observe any evidence of nicotine synthesis.

A particularly voluminous literature abounds on the biosynthesis of the tropane alkaloids. After Robinson (208) called our attention to the possibilities of attaining tropinone with the help of succindialdehyde, methylamine, and acetondicarbonic acid, and after Schöpf (225) had found corresponding syntheses *in vitro* under "physiologic" conditions, Cromwell (35) and James (119, 121) reported that by feeding detached leaves or injecting intact plants (*Atropa, Datura*) with arginine, ornithine, and putrescine, hyoscyamine increased. James (121) discovered that proline as well as histidine, leucine, glycine, and valine do not show such an effect. But these results are not completely convincing because occasionally sucrose alone

causes a considerable increase in the alkaloid content. James believes that ornithine and putrescine are changed into succindialdehyde, but Trautner (246) prefers diketoadipinic acid as the real precursor.

Located chiefly in the root system but found also in etiolated shoots, Cromwell (35) found an enzyme capable of oxidizing putrescine with the formation of ammonia and an aldehyde. Putrescine has been isolated in small amounts from leaves and upper stems of *Atropa* and *D. stramonium*. Fuller & Gibson (69) compared the alkaloid content in different organs of *D. tatula* with the arginase activity. Both values increase simultaneously and extensively until about the tenth week. The authors conclude that these relationships tend to support the theory that the first step in the biosynthesis of the tropinone alkaloids is the hydrolytic action of arginase on molecules containing arginine.

But the investigations of James and Cromwell could not be confirmed by Diaper, Kirkwood & Marion (50). They fed putrescine (1,4-C^{14}) to *D. stramonium* and did not obtain radioactive hyoscyamine. In a recent publication Leete, Marion & Spenser (152) once more stated their opinion on this important problem. They have tested this hypothesis by feeding radioactive ornithine (α-C^{14}) to roots of mature *D. stramonium*. After a week, almost all the activity had disappeared from the nutrient solution. Paper chromatography of the alkaloids showed radioactivity to be present in hyoscyamine, but little or none in scopolamine. The activity of hyoscyamine is present in the bridgehead carbons (1 and 5) of the tropine and none in the tropic acid. From this it follows that ornithine is indeed the precursor of the pyrolidine ring of tropine. Putrescine is apparently ruled out as an intermediate between ornithine and tropine. It is possible that the scopine of scopolamine is produced from a hypothetical hydroxyornithine.

In this connection we should refer to some striking relations between the existence of nicotine and the tropinone alkaloids in the genus *Duboisia* [Barnard (5)]. Until quite recently *D. myoporoides* has passed for a scopolamine-hyoscyamine specimen. But Hills, Bottomley & Mortimer (103) made the important discovery that descendants from New Caledonia contained nicotine as well as scopolamine. They presume that this chemical strain might perhaps be the primitive form of *Duboisia* and that for the first time a coexistence of nicotine and tropinone alkaloids has been found. The phylogenetic conclusions, however, seem rather uncertain. For Wahl has shown that within the family Solanaceae nicotine is widely distributed. According to our own experiences it appears to be a "Leitalkaloid" of this family. Romeike (211) found, in the shoot of *Atropa*, that a large amount of mydriatic alkaloid was produced when the stock was *Nicotiana*. This leads to the assumption that *Nicotiana* substances form the precursors of the tropinones.

Still more important may be the mutation experiments in which *Datura* strains, containing only traces of nicotine, give rise to new forms in which the nicotine has increased considerably [Mothes, Romeike & Schröter (183)].

It is entirely possible that nicotine and the tropinones have the same precursor. Hills, Bottomley & Mortimer (103) discuss their results with *Duboisia* in a similar manner. In this connection Mortimer (165) points to the simultaneous existence of D- and L-nornicotine, the L-form predominating in tobacco, the D-form in *D. hopwoodii* [Hicks & Sinclair (98)]. Mortimer reasons that this argues against a direct origin of the pyrrolidine ring from L-proline. In *D. hopwoodii*, however, only the L-form exists, which leads to the conclusion that biologic methylation is only possible with L-nornicotine. Finally, Mortimer concludes that the coexistence of hygrine or cuscohygrine and tropane alkaloids in *Erythroxylon*, *Convolvulus*, and *Scopolia* proves that the synthesis of the tropinones takes an analogous course to that of the pyridine ring from tryptophan. As early as 1917, Robinson (208) called attention to the structural similarity of hygrines and tropanes. And it is interesting to note, on the other hand, that in Cinchona, in addition to the typical quinoline nuclei alkaloids some have been found with indole nuclei [quinamine, Kirby (132)]. Robinson (209) has raised certain objections to the ideas of Mortimer, and the above-cited investigations of Leete, Marion & Spenser support these objections. Of great importance, in these theoretical considerations is the discovery of cuscohygrine in the seedling phases of different mydriatic Solanaceae by van Haga since this base had already been found in *Scopolia* [Henry (97)].

The fact should be noted that L-pipecolic acid may be synthesized by the rat [Rothstein & Miller (211a)] and by *Neurospora* [Schweet, Holden & Lowy (229a)] from lysine.

Genetics of Alkaloids

Few investigations concerning the heredity of polygenically restricted alkaloid characteristics [Steinegger (238)] have been made in recent years. We are obliged to recall the writings of Klawitter & von Sengbusch (136) on the genetics of the character "bitterness" in lupines, and on the older reports about tobacco by Kostoff (140); Schmuck (221); Smith & Smith (233); Kuzmenko & Tikhvinskaja (147) which are of small interest in this connection. In crosses of alkaloid plants, special heterosis effects with respect to alkaloids are not positively known [Reich (204)].

The increase of alkaloid in polyploids is emphasized repeatedly [Rowson (212); Kawatani, Ohno & Imasaki (130)], although by no means is it always observed [cp. Koelle (139a); Steinegger (241)]. The contention, held by Rudorf & Schwarze (213), is that the difference between more root substance as compared to less leaf is responsible for the increase. According to Steinegger (238 to 241), these results are still rather uncertain. He does not find a characteristic difference in the root-shoot relation in tetraploid and diploid forms. Also the smaller seed setting of the tetraploids cannot explain the increased content of the foliage. Prasad (197) demonstrated in 1948 that a diminution in the quantity of fruits by no means causes an increase of the alkaloid content of the leaves. Steinegger reports a general increase of

metabolic activity in polyploids. On the basis of the polygenic control of alkaloid synthesis, he sees the definite possibility of obtaining more valuable alkaloid plants by hybridization of polyploids followed by selection. Shortly the question of vegetative hybridization must be examined. Braslawskaja (18) grafted tomato upon *Atropa*, and from the tomato she cultivated seed descendants which microchemically gave alkaloid reactions with KI or tannin. Tomato controls were not analysed, the amount of material was rather small, and the alkaloids were not identified. Tuschnijakova (248) offers numerous results on the inheritance of alkaloid characteristics obtained by grafting. She states that the fruits of the first seed descendants of the tomato grafted upon *N. rustica* contain 0.73 per cent nicotine, the second generation 0.35 per cent, and the third 0.2 per cent. Inversely, the seed descendants of originally alkaloidal species which, on alkaloid-free root had become alkaloid-free (e.g., *Atropa* upon tomato), are said to become gradually alkaloidal again. Glustschenko (73) has assembled the literature in question. We have very often repeated these experiments but have found nothing which might correspond to a hereditary or acquired alkaloid character, or to a permanent modification [Mothes & Romeike (181)]. Also, the investigations of Kerkis & Pigulevskaja contradict Glustschenko's opinions; in fact, so do the results of Iljin (114). He found that nicotine-containing leaves of tomato grafted upon tobacco quickly lose their nicotine and return to their normal state when isolated and made to form roots.

The Taxonomic Significance of Alkaloids

Our knowledge of the distribution of alkaloids within the plant families as well as in the different phytogeographical districts of the earth is still incomplete. Only in a few cases has anyone begun systematically to examine a whole flora for its chemical characteristics, as is being done in the Soviet Union [see Ssokolov (235) and Henry (97)], and in Australia [Webb (259)]. The latter reports on the alkaloid content of 1040 species of Angiosperms of which 206 species from 108 genera and 37 families contained quantities worth mentioning. Since the alkaloids occur only in traces, this number will probably be raised considerably.

Reference must be made to the writings of Rygh, Rygh & Laland (214) concerning the occurrence of narcotine in traces in oranges, tomatoes, cabbage, and potatoes, although it was looked upon as specific for the Polycarpicae and Rhoeadales or to the occurrence of nicotine which was considered to be specific for the genus *Nicotiana* alone, and which now has been found not only in all Solanaceae analysed (Wahl called attention to it), but also in Asclepiadaceae, Compositae, Crassulaceae and, above all, in Equisetaceae and Lycopodiaceae [Henry (197); Manske & Holmes (155); Pal & Narasimhan (193); Marion (156); Mothes (172, 173); Schröter (228a)].

The reason why nicotine exists in *Nicotiana* in large quantities, but in other Solanaceae, only in traces, may be found in mutation experiments [Mothes, Romeike & Schröter (183)]. It is a simple matter to produce

mutants of *D. stramonium* which contain unusual quantities of nicotine although not as much as the species of the genus *Nicotiana*. These mutants are slow to develop. The fact that such races are not to be found in nature may be easily understood from grafting experiments. *Datura* species, *Atropa* and other Solanaceae grafted upon *Nicotiana* rich in nicotine, show severe damage of the leaves of the scion. It may be asserted that the genera *Datura* and *Atropa* are not incapable of developing species with high nicotine content, but that these species are not able to survive. *Datura* is not distinguished by the absence of nicotine or by an incapacity to produce it but by an incapacity to maintain it.

In a similar way may be discussed the relationship between predominant and minor alkaloid which may differ widely from species to species. The genus *Nicotiana* is well-analysed. According to the early concepts, nicotine is a characteristic of *N. tabacum* and nornicotine of a number of wild species [Smith & Smith (233); Markwood & Barthel (157); Borozdina (12)]. But to-day we know several strains of *N. tabacum* which contain nornicotine predominantly in the shoot. Mutation experiments with *N. rustica* have shown that the nornicotine and anabasine content can be increased. The question why anabasine is dominant in *N. glauca* but not in *N. tabacum* races must still be answered. It is conceivable that such anabasine mutants possess a limited vitality like the nicotine mutants of *Datura* [Mothes, Romeike & Schröter (183)].

Extensive analyses should be made of populations of wild plants if we are to have a clearer concept of the species-specificity and race-specificity of the occurrence of alkaloid. Possibly, we must admit that certain cultivated plants, beginning with the originals, developed on a genetically restricted basis without reflecting the real variety of the chemical characteristics of any one species. There have been many surprises connected with the occurrence of alkaloids in the past. Reference is made, for instance, to a report by Pyriki (200) whose findings, in contrast to the hitherto published results of the occurrence of L-anabasine in *N. glauca*, revealed a racemic form in one strain which amounted to 90 per cent of the total alkaloid.

Hence, the chemical differentiation of the species and genera may be widespread. Recall, once again, *Duboisia*, in which different species and geographical strains contain, alternately and predominantly, nicotine, nornicotine, hyoscyamine, or scopolamine [Barnard (5); Bottomley, Nottle & White (13); Hills & Rodwell (101, 102); Hills & Kelenyi (104); Hills (100)]. These investigations must certainly not yet be regarded as finished. Most important of all, there must be a decisive and thorough clarification of the extent to which environmental relationships explain the analytical results.

These questions are a small part of the overall systematics of the alkaloid character. Just as one must be cautious in interpreting isolated observations (as the examples nicotine and nornicotine have shown) so also must one not overlook the fact that several alkaloids seem to have a systematically incon-

testable occurrence. Perhaps the frequent convergence of a chemical character means that by relatively few transformations the various alkaloids stem from a generally distributed substance. In this way might be understood the frequent appearance of the carboline-type of the berberine, etc. [Mothes (173)].

It seems remarkable that in the literature there is again and again to be found the hint that Cryptogams are poorer in secondary plant substances, especially alkaloids, than higher plants. On no account can the thesis of Paech (191) be maintained, namely, that in Pteridophytes and Gymnosperms, in contrast to the Angiosperms, alkaloids are to be found only sporadically and that the ergot alkaloids are peptides but not true alkaloids.

The lower plants, especially fungi and lichens, seem to be real arsenals of secondary plant substances, whereas the submerged living forms are likely to be poorer in them. The research on antibiotics has impressively revealed the varied chemical power of the cryptogams. Only systematic analyses can lead to a conclusive judgment about the occurrence of alkaloids.

Ergot alkaloids are not only doubtlessly found in sclerotii but also in saprophytic cultures [Rochelmeyer (210)]. Alkaloids of a similar type also seem to be found in sclerotinia [Gradnick (76); Satomura (216)]. Hegnauer (92) has stated that substances related to the alkaloids seem to be the fusarinic acid from *Gibberella fujikuroi*, the aspergillic acid from *Aspergillus flavus*, and pikrorocelline from *Lehenes*. Bracken, Pocker & Raistrick (17) report the occurrence of the complicated chinoline derivates, cyclopenine and viridicatine, in Penicillium species. Neither must we overlook substances like prodigiosine, a tripyrylmethine pigment from *Bacillus prodigiosus*, on the biosynthesis of which Hubbard & Rimington (108) report, and a pigment occurring in yeast (*Torula*) noticed by Kluyver, van der Walt & van Triet (139) as the ferric complex of a diketopiperazine.

We must also mention the occurrence of bufotenine in *Amanita* species, above all because here one of those rare relations to the animal organism exist [Wieland & Motzel (265)]. That plant bases are not found more frequently in the animal is, surely, not only the proof of a more restricted metabolism in the animal as a consequence of its greater specialization, but also the manifestation of a better functioning excretion mechanism. We do, however, find secondary plant substances in the animal, particularly in the secretory glands of the cuticle or similar parts, e.g., in the toad where, beside bufotenine, digitaloidal heart active poisons occur.

The Role of Alkaloids

There is no need to elaborate on the fact that teleological interpretations of the alkaloids have had few satisfying results. Alkaloids generally offer no protection against animals and parasitic fungi [Ahmed & Fahmy (2); Mothes (170); James (123)]. This does not preclude the special cases wherein alkaloids may be significant in safeguarding against certain parasites. I refer

to Greathouse & Watkins (77) who report that *Mahonia*, in districts where the root rot *Phymatotrichum* is general, is not attacked and that berberine stops the growth of this fungus. Kuhn & Löw (143) and Kuhn & Gauhe (142, 144) published investigations which make it probable that certain solanidine variants, e.g., demissine, are the cause of the resistance in certain wild potatoes against *Leptinotarsa decemlineata*. From a South American small-fruited tomato, Doolittle & Fontaine (52) isolated a solanidine which has an antibiotic effect against the stimulant of the Fusarium wilt of tomato (*Fusarium oxysporium*).

Without any doubt alkaloids are physiologically active. Numerous Solanaceae, which regularly contain traces of nicotine when grafted upon nicotine-enriched tobacco species, show the greatest disorder—a bleaching out of the chloroplasts. Nicotine was shown to be the disturbing substance, which means that root-specific substances may be the reason for the incompatibility of graft-partners [Mothes & Romeike (182)]. In this connection, note that the composite *Zinnia elegans*, grafted upon *Nicotiana* root grows and flowers as well as on its own root. Schröter (228a) demonstrated that ungrafted *Zinnia* contains relatively great quantities of nicotine and therefore seems to be accustomed to the alkaloid of the *Nicotiana* root.

Thus, one cannot expect that nicotine-enriched *Atropa* species should be able to exist, even though *Atropa* always contains small quantities of nicotine. Therefore, the alkaloid content is not always determined by the capacity of alkaloid synthesis itself but also by the sensitivity of the plant to such alkaloids. Thus, it could be shown that *Datura* forms can be induced which contain more than traces of nicotine. They have a weakened growth (Mothes, Romeike & Schröter). In *Atropa*, *Datura*, and *Cyphomandra* the alkaloid nicotine destroys the chloroplasts. The fact that tobacco contains nicotine in higher concentrations means not only a special biosynthetic capacity but a higher resistance to nicotine.

The preceding suggests how difficult, if not impossible, a general theory of alkaloid function may be. As scions of alkaloid plants are able to grow almost without any alkaloid. But we do not know a single positive case that proves their being totally free of alkaloid. In mutation experiments no mutants free of alkaloid have yet been achieved (Mothes, Romeike & Schröter). It is probable that they occur no more frequently than chlorophyll-defective mutants. That they have not been discovered might be caused by the fact that alkaloids or the process of their production are necessary for existence. Like Cromwell (33, 34), this reviewer ascribes little importance to those hypotheses which consider alkaloids to be reserves of nitrogen metabolism comparable to asparagine [Schmuck (221)].

Dawson (44) says that nicotine increases nitrate absorption and possibly nitrate reduction in the roots of tobacco plants. Schmid (218) and Trapp (245) also find striking relations between nicotine and nitrate nutrition. These are important considerations for a physiological theory.

The possibility of alkaloids functioning in transmethylations was already

stressed above. The discovery of Frankenburg (66) of the occurrence of nitrogen oxide compounds in nicotine, and of Areškina (3, 4) of similar oxides in the alkaloids seneciphyllin and platyphyllin isolated from *Senecio platyphyllus*, inaugurate new possibilities for a participation of alkaloids in oxidation-reduction processes. During vegetative growth Areškina finds 80 per cent of the alkaloid in oxides while during ripening, no nitrogen oxides are found at all.

Another possible function of alkaloids, especially in the root tip, may be an antiauxin activity. Some observations indicate this, e.g., the rôle of nicotine as florigen in soybeans [Fisher & Loomis (64)] as well as manifold growth inhibition of the scions of alkaloid-containing plants on alkaloid-free stock.

Totally unexplored is the question of the rôle alkaloids play in the soil into which they may be leached from the leaves or excreted by the roots. The possibility exists that they represent an ecologically important factor.

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THE NUTRITION OF PLANT TISSUE CULTURES[1]

By R. J. Gautheret[2]

Laboratoire de Biologie Végétale, Faculte des Sciences de Paris, Paris, France

Introduction

The first investigators to attempt the cultivation of tissues believed that the nutritional requirements of animal cells must differ profoundly from those of plant cells. The fact that animals demand a complex type of nutrition, including all the classes of biochemical compounds (proteins, carbohydrates, lipids) together with biological catalyzers such as vitamins, suggested the utilization of natural media in attempting to cultivate these cells. With plant tissues, on the contrary, one looked to simple mineral solutions such as those allowing the development of whole plants, with the addition, however, of a sugar and sometimes of some stimulants of cell division.

The exploitation of these two ideas led to a double success. By using media composed of blood plasma and chick embryo extracts, Carrel (48) in 1912, obtained for the first time the indefinite culture of animal cells. The indefinite culture of plant tissues was obtained by White (290), Gautheret (76), and Nobécourt (223) by means of entirely synthetic media containing mineral solutions (Knop, White), glucose (Gautheret, Nobécourt) or sucrose (White), vitamin B_1 (White, Gautheret, Nobécourt), glycine (White), and cysteine or indoleacetic acid. The sharp difference between the general constitution of the media employed for animal and plant cells had several consequences. Thus, it was not possible to define exactly the nutritional requirements of animal cells. The nature of the specific factors for cell division or trephones contained in embryo extracts is still insufficiently known [Fischer (71)]. For that single reason the culture of animal tissues has not yielded as yet exact physiological results.

Plant tissue culture specialists are in a very different situation, as they had at their disposal from the beginning definite substances capable of maintaining cell proliferation. Their first successes resulted from the methodical utilization of auxins [Gautheret (75), Nobécourt (223)], and vitamin B_1 [Nickell (213)], or pantothenic acid [Morel (192)]. For a long time, the list of these division-inducing substances remained very short. It was increased when certain seed and tissue extracts were found to possess stimulatory properties. The most interesting of these natural products is coconut milk [van Overbeek, Conklin & Blakeslee (232); Caplin & Steward (44)]. Its use has permitted the preparation of media much more active than those containing auxins; but the nature of the stimulatory substances contained therein has not yet been entirely elucidated.

[1] The survey of the literature pertaining to this review was concluded in 1954.

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At the same time that natural media were beginning to be employed in plant tissue culture, research on animal tissue nutrition moved in the opposite direction. An effort was made to replace the primitive media of indefinite constitution with complicated but completely synthetic solutions. By patiently using the trial and error method, Fischer *et al.* (72) and White (296, 299) were able to propose media containing mineral salts, glucose, and numerous amino acids and vitamins. These media allowed the proliferation of animal tissue for periods which, though sometimes prolonged, were always limited. Thus, the study of the nutrition of plant tissues is far in advance of that of animal tissues. The time is ripe, therefore, for a general review of the subject.

We shall examine the mineral nutrition, the carbohydrate nutrition, the action of stimulatory substances, and, finally, certain particular points concerning, for example, the relation between the physical state of the medium and proliferation, or the modifications in nutritional requirements which occur in the course of the culture.

Mineral Nutrition

We shall consider first the composition of the solutions employed by the founders of plant tissue culture; then the refinements proposed as a result of methodical investigations; and finally we shall examine the possibility of replacing certain ions by various mineral or organic compounds possessing an equivalent nutritional value.

Mineral solutions used by the early investigators.—The investigators who first succeeded in cultivating plant tissues did not attempt an exact determination of the mineral needs of their material. They empirically adopted solutions successfully used for the culture of entire plants or of isolated organs. White, for example, utilized successfully for numerous tissue strains, the medium which he had developed in his root culture investigations (292) and this medium happened to be satisfactory for tissues. It contains (Table I) six macroelements indispensable for the development of higher plants (N, S, P, K, Ca, Mg), two macroelements, the role of which is doubtful (Na and Cl) and five microelements (Fe, B, Mn, Zn, I).

Gautheret (76) and Nobécourt (223) simply used Knop's solution diluted to one-half, plus the microelements recommended by Berthelot (7). This mineral medium differs qualitatively from that of White by the absence of two macroelements (Na and Cl) and the presence of five supplementary microelements (Cu, Ni, Co, Ti, Gl).

There are also quantitative differences. The most important concerns phosphorus which is almost six times more abundant in Gautheret's solution than in White's (Table I). These two mineral solutions were used as such during some ten years.

Improvement of the mineral solutions.—In 1946, Hildebrandt, Riker & Duggar (136) undertook the first experimental study of the nutritional requirements of tissue colonies. Their experiments bore exclusively on tumor

TABLE I

COMPOSITION OF THE MINERAL SOLUTIONS USED FOR PLANT TISSUE CULTURE [ACCORDING TO HELLER (126)]

A. Macroelements
(Concentration in milimoles per liter)

Ions	Gautheret's Solution	White's Solution	Solutions of Riker *et al.*		Burkholder & Nickell's Solution (*Rumex* and *Melilotus*)	Heller's Solution
			Tobacco (tissues of the tumors from the hybrid *Nicotiana langsdorfii* × *N. glauca*)	Sunflower (crown gall tissues)		
NO_3^-	5.5	3.2	4.2	8.4	8	7.1
K^+	2.2	1.7	1.7	3.3	12	10.0
Ca^{++}	2.1	1.2	1.7	3.4	3	0.51
Mg^{++}	0.5	3.0	0.7	2.9	2	1.03
$SO_4^=$	0.5	4.4	6.4	3.6	1	1.03
$PO_4H_2^-$	0.9	0.14	0.24	1.0	8 for tumor tissues of viral origin 1 for the other tissues	0.91
Na^+	—	3.0	11.7	1.7	—	8.0
Cl^-	—	0.9	0.9	1.8	8	11.0

B. Microelements
(Concentration in grams per milliliter)

Elements	Gautheret-Berthelot's Solution	White's Solution	Solution of Riker *et al.*		Burkholder & Nickell's Solution (*Rumex* and *Melilotus*)	Heller's Solution
			Tobacco (tissues of the tumors from the hybrid *Nicotiana langsdorfii* × *N. glauca*)	Sunflower (crown gall tissues)		
Iron	7.0×10^{-6}	7.0×10^{-7}	8.0×10^{-6}	1.0×10^{-6}	5×10^{-7}	2.0×10^{-7}
Boron	4.5×10^{-9}	2.7×10^{-7}	6.6×10^{-8}	5.3×10^{-7}	1.0×10^{-7}	1.8×10^{-7}
Manganese	2.5×10^{-7}	1.7×10^{-6}	1.1×10^{-6}	1.1×10^{-6}	1.0×10^{-7}	2.5×10^{-8}
Zinc	1.1×10^{-8}	6.1×10^{-7}	1.4×10^{-7}	6.8×10^{-8}	3×10^{-7}	2.3×10^{-8}
Iodine	1.9×10^{-7}	5.7×10^{-7}	2.3×10^{-6}	2.8×10^{-7}	—	7.6×10^{-9}
Copper	6.4×10^{-9}	—	—	—	1.0×10^{-7}	7.7×10^{-9}
Molybdenum	—	—	—	—	1.0×10^{-7}	—
Nickel	6.2×10^{-9}	—	—	—	—	7.5×10^{-9}
Cobalt	6.2×10^{-9}	—	—	—	—	—
Titanium	4.2×10^{-8}	—	—	—	—	—
Glucinum	2.5×10^{-9}	—	—	—	—	—
Aluminum	—	—	—	—	—	7.5×10^{-9}

tissues (sunflower crown gall and spontaneous tumors in the hybrid *Nicotiana langsdorfii* × *N. glauca*) as, at that time, normal tissues were still not cultivated in the United States. They adopted the classical method of the triangle which permits the determination in a single series of experiments of the optimum concentration of any three salts, for example, $MgSO_4 \cdot 7H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, and Na_2SO_4. This method has the advantage of quickness but lacks flexibility, as the sum of the concentrations of the three salts is invariable. As a result of their investigations, Hildebrandt, Riker & Duggar (136) made some quantitative improvements in White's solution (Table I). They increased, for example, the phosphate-ion concentration, particularly for the sunflower crown gall tissue.

Three years later, Burkholder & Nickell (36) also conducted researches on the mineral nutrition of tumor tissues. They turned to the tumors caused on sorrell by the virus *Aureogenus magnivena*, a tissue which had been cultivated by Black in 1944 (18). In the first series of experiments they, too, adopted the triangle method, but subsequently varied independently the concentration of each ion. They used as a starting point one of the media of Hildebrandt, Riker and Duggar and studied the effect of the ions $PO_4H_2^-$, NO_3^-, and $SO_4^{=}$. Their very well-conducted work suggested the need for a large increase in phosphate-ion concentration, which was upped to 8 millimoles per liter, i.e., nearly 60 times that used by White (Table I).

Nickell (217) has found that virus tumor tissue obtained from *Melilotus officinalis* also has abnormally high phosphate-ion requirements, while other tissues from the same species (normal, crown gall, and auxin-habituated tissues) do well with a concentration of phosphate ions of the order of one millimole per liter. The special behavior of virus tumor tissues is probably due to the presence of the phosphorus-rich virus.

The works mentioned above did not cover the whole problem of mineral nutrition, as it was performed on only a few tissues, most of them of a pathological nature. More general investigations were then undertaken in France by Heller (124 to 128).

Having found that the agar usually included in the nutrient media includes important amounts of utilizable mineral elements (Ca, Mg, various microelements), Heller turned to liquid media. This raised the difficulty that most tissues are unable to grow in liquid. The difficulty was solved by de Ropp (253) and later by Caplin & Steward (45) by using an apparatus capable of placing the colonies alternatively in the nutrient solution and in air. Heller (124) used a simpler device consisting of an ash-free filter paper support upon which the explant is maintained at a convenient level.

From the very first he verified that plant tissues are very senstitive to mineral deficiencies. If, for example, one transfers to media devoid of an essential macroelement (N, S, P, Ca, K, or Mg) death occurs after the first to the second two-month passage (Table II). Before causing death, some deficiencies provoke disturbances in the carbohydrate metabolism. Thus, a deficiency of nitrogen induces anthocyanin formation in Virginia creeper

tissue (126). This has been confirmed by Slabecka-Szweykowska for vine tissues (272). But one must not neglect the influence of other factors such as sugar concentration, osmotic pressure, illumination, etc., which have been stressed by Slabecka-Szweykowska in her experiments on vine tissue culture. Deficiency of certain microelements (Zn, Fe, Mn, B, Cu) is similarly followed by deficiency phenomena but these, of course, manifest themselves less clearly than those of the macroelements. Heller also found that certain macroelements, often considered inactive, such as Na, are not necessary but stimulate proliferation appreciably. The same effect is exerted by various microelements such as Ni, Al, and I.

TABLE II

MINERAL DEFICIENCIES IN CARROT TISSUE CULTURES PREVIOUSLY GROWN ON GAUTHERET'S MEDIUM [ACCORDING TO HELLER (126)]

Elements omitted from the nutrient medium	Number of passages necessary to obtain death of the tissues
Macroelements	
N	1
K	1 to 2
Ca	2
Mg	2
S	2
P	1 to 2
Microelements	
Fe	4
B	3
Mn	4 to 5
Zn	3
Cu	4

In the course of his experiments, Heller established that tissues react markedly to variations in ion concentrations. For most elements (macro and micro) well marked optima are observed, but surpassing these levels rarely have toxic effects, unless exaggerated amounts are used. In conclusion, Heller proposed in 1953 (126) a new mineral solution which yielded significantly better results than those obtained with the classical media of Gautheret and White.

If one looks at Table I, which shows the composition of the different mineral solutions used in tissue culture, several conclusions can be made. First, the systematic studies of Nickell & Burkholder (36) and Heller (126) have conclusively established that the old media are, as a whole, poor in potassium and that White's solution should be enriched in phosphorus. On the contrary, Heller demonstrated that the calcium concentration should be low.

It must be understood that the mineral requirements of a given tissue are not fixed but depend on its physiological state. The investigations of Heller (126) have shown, for example, that vigorous colonies of carrot tissue demand more potassium than slowly proliferating ones. Calcium behaves contrariwise, i.e., fast growing tissues require less calcium than slow growing ones. It is thus seen that, in principle, the mineral composition of nutrient solutions should be adapted to each particular situation.

Nevertheless, the solution of macroelements proposed by Heller is satisfactory for most tissues. Trials on eleven different strains, normal and tumorous, have shown that, other conditions being equal, the use of this solution allows an average twofold increase in the rate of proliferation over that obtained with Gautheret's medium and a threefold increase over that given by White. It is certainly not a panacea; thus in tobacco tissue (crown gall and auxin-habituated) it causes a more or less total necrosis of the colonies. On the contrary, the formulas of Gautheret (76), White (288) and Riker *et al.* (136) give satisfactory results.

Having considered the macroelements, let us now compare the different solutions with respect to their microelement composition. As one glances through Table I, one finds that, on the whole, Nickell, and especially Heller, have considerably reduced the concentrations of microelements from those used by their predecessors which, in some instances, were toxic. With boron, however, it is advisable to increase the concentration over that recommended by Gautheret. It should be noted that Heller uses aluminum, neglected by other authors. The superiority of Heller's microelement solution over that of Berthelot-Gautheret was shown by an experiment on carrot tissue. Its use trebled the growth rate. This result, however, was obtained in liquid media. On solid media, the phenomena of deficiency and toxicity are attenuated and the advantage of a well-adapted microelement solution is not so marked.

Replacement of certain ions.—The above-mentioned experiments consisted essentially in quantitative modifications, but brought little changes in the qualitative composition of the mineral solutions. The investigators then considered whether certain elements particularly nitrogen and sulfur, could be used in different mineral or organic forms. This type of work was inaugurated in 1948 by Riker & Gutsche (247) who attempted to replace NO_3^- with NO_2^- or NH_4^+.

They established that the nitrite ion cannot substitute for the nitrate ion. Low concentrations of nitrite lack sufficient nutritive value to allow growth, while higher concentrations are toxic. The ammonium ion is less toxic, but its nutritive value is well below that of nitrate. Burkholder & Nickell (36), working with tumors produced by the virus *Aureogenus magnivena*, have similarly verified the superiority of NO_3^- over NH_4^+, and Heller confirmed this for normal tissues (126), showing that this phenomenon could not be explained by either a pH effect nor by the effect of the ammonium ion on carbohydrates (127).

The replacement of the nitrate ion by organic nitrogen compounds has

been more successful. First Riker & Gutsche (247), then Frank, Riker & Dye (73) and finally Nickell & Burkholder (218) have found that urea allows excellent growth and is sometimes even superior to nitrate. Amino acids behave in various ways. The work of Riker & Gutsche (247) on sunflower crown gall has shown that many cannot be utilized (for example, histidine, leucine, tryptophan, tyrosine, etc.). But others, such as alanine, glycine, arginine, glutamic acid, aspartic acid (and asparagine) can replace nitrate to a certain extent. Finally, certain complex substances such as peptone, casein hydrolysate, and yeast extract are also utilized by sunflower crown gall tissue.

With tissues from tumors produced by the hybrid *N. langsdorfii*×*N. glauca*, Frank, Riker & Dye (73) have obtained somewhat different results. In general, the ability of these tissues to assimilate amino acids is less than that of sunflower crown gall tissues. They can, however, use glutamic acid and alanine to a certain extent. Nickell & Burkholder (218) have examined the possibility of utilization of amino acids by tissues from the tumors induced on sorrel by *A. magnivena*. Only glycine, aspartic acid, and proline proved to be efficient nitrogen sources. Nickell and Burkholder have furthermore established that by combining aspartic acid with nitrates, a stimulation is observed, which results from the fact that the amino acid selectively promotes cell proliferation. We shall return to this later when examining the effect of substances stimulating cell division on tissue culture growth. Riker & Gutsche (247) obtained equally interesting effects of combinations of aspartic acid and nitrate. By cultivating sunflower crown gall tissue on media containing the optimum level of nitrate and increasing concentrations of aspartic acid, they observed an inhibition which, starting at a concentration of the order of 0.001 *M*, became total at 0.004 *M* and disappeared in part with concentrations of 0.016 *M* or 0.064 *M* of aspartic acid. Similar phenomena were observed when sunflower crown gall tissues were cultivated with nitrates and alanine or glutamic acid; but nothing similar could be detected using genetic tumors of tobacco [Frank, Riker & Dye (73)] or with the virus-induced tumors of sorrel [Nickell & Burkholder (218)]. One is tempted to explain this singular effect of glutamic and aspartic acids and alanine on crown gall tissue cultures as an inhibition of nitrate absorption; it would thus be related to mineral nutrition.

This inhibition would not occur, of course, at very low concentrations of amino acid and tissues would utilize the nitrate for growth. At somewhat higher concentrations, still destitute of nutritive value, it would begin to show up and the growth of the cultures would be hindered. But, if one still increases the amino acid concentration, these would now serve as nitrogen sources with consequent resumption of growth, in spite of the suppression of nitrate assimilation. This tempting explanation does not hold after the demonstration by Eberts, Burris & Riker (69) that the three amino acids do not impede nitrate assimilation. Their work also invalidates any hypothesis involving interference with transamination.

As with nitrate, attempts have been made to replace $SO_4^{=}$ with other

mineral or organic forms. The first work was done in 1950 by Nickell & Burkholder (218) who used the tumors produced on sorrel by *A. magnivena*. They established that bisulfite had the same nutritive value as sulfate; sulfides are similarly utilizable but not so well. Among the organic sulfur compounds tested, cysteine was as effective as sulfates, while its oxidation product, cystine, as well as glutathione and methionine, showed no nutritive value. Heller (unpublished data) has carried out analogous trials on normal carrot tissue. He found that sulfites, sulfides, cysteine, glutathione, and methionine are as good as sulfates. Taurine, however, cannot be utilized on account of its toxicity. It appears then, that the numerous sulfur compounds are satisfactory.

Present orientation of investigations in the mineral nutrition of tissue cultures.—The studies which we have just examined have clarified the problem of the mineral nutrition of tissue cultures and have broadened its scope by showing its relationships to organic nutrition. Their principal merit lies in having rendered considerable service to technicians by improving the nutritive media.

Having attained this result, one now looks to other problems. Thus, one begins to consider the relation between the composition of the external medium and the mineral composition of the tissues. Preliminary experiments made by Heller (127) have revealed how difficult this study can be, but one would be wrong in being disheartened, as the question is very important. The most classical studies of ionic interaction have not been always successful (127). Heller has clearly shown antagonism between Na and K, and Ca and Mg, but in the case of the pairs P—Ca or P—Mg the interactions were obscure. These results are surprising since it is known that plant tissue cultures react intensely to modifications in the mineral constitution of nutrient solutions. But we must repeat that we are dealing here with a new avenue of research and it would be premature to draw conclusions.

It should be pointed out that the use of labeled compounds with *in vitro* cultures appears to be promising. The first work of this type has recently been undertaken by Ball (4). This investigator cultivated *Sequoia* tissues in media containing P^{32}-labeled phosphoric acid and S^{35}-labeled sulfuric acid. These radioactive elements accumulated in the tissues and Ball determined their localization by means of radiohistoautographs.

Finally, Eberts, Burris & Riker (69) have recently shown that tissue culture and the technique of labeled compounds can be fruitfully used in conjunction with chromatographic analysis. They have thus shown that the growth inhibition caused by certain concentrations of aspartic and glutamic acids and alanine is not due to an inhibition of nitrate assimilation. Investigations of this type will certainly multiply in the near future.

Carbon Nutrition

The first investigators to carry out *in vitro* cultures believed that tissues from chlorophyllous organs would possess the faculty of growing without an organic carbon source. Experiments did not confirm this belief since tissue

colonies are not carbon-autotrophic. Even if the primitive explant is rich in chlorophyll, it becomes more or less completely depigmented during culture and ceases photosynthesis. Plant tissues must be cultivated, then, in sugar-containing media.

Over many years, glucose [Gautheret (80)] or sucrose [White (292)] have been empirically used in concentrations ranging from 2 to 5 per cent. These two sugars provide for good growth. Subsequently, attempts have been made to determine the other carbon sources suitable for tissue colonies. Lastly, some investigators have examined the transformations undergone in the cells by the absorbed sugars. We shall examine both these questions.

The carbon sources studied can be classified into three groups: carbohydrates, alcohols, and organic acids. We shall examine the results obtained with these three types of substances.

Carbohydrates.—The first work on the carbohydrate nutrition of plant tissue cultures was done in 1941 by Gautheret. He determined the optimum concentration of sucrose for normal carrot tissue (79); then he compared its nutritive value with that of many other sugars used at the same molar concentration (84). The quantitative results obtained from many trials are summarized in Table III, in which the relative efficiency of each sugar is indicated by the number of crosses. Sucrose is the best carbon source followed by glucose, maltose, and raffinose; fructose and galactose come next, then mannose and lactose which are clearly less efficient. Pentoses and polysaccharides cannot be utilized by normal carrot tissue.

Analogous experiments were done some years later in the United States. In 1945, Hildebrandt, Riker & Duggar (136), studying the growth of sunflower and tobacco tumor tissues in media supplemented with various sucrose concentrations, found an optimum of the order of 1 per cent. Hildebrandt & Riker (133, 134) cultivated several crown gall tissues and genetic tumor tissues of tobacco in media containing different sugars. Their results confirm, in general, those of Gautheret with regard to the nutritive value of sucrose, glucose, and fructose (Table III). They also found that pentoses were not assimilated and that certain sugars behaved differently according to the nature of the tissue. Thus, galactose was an excellent carbon source for *Vinca rosea* crown gall tissue, but it was not utilized by other tumor tissues. Similarly, certain tissues grew more or less satisfactorily on media with mannose or lactose, while others were incapable of utilizing these sugars. Henderson (129) has made an analogous comparison, though on a smaller scale, using sunflower tissue. He observed that glucose was better than fructose and that sucrose was still better (Table III). Finally, Ball (personal communication) studied the effect of several sugars on normal *Sequoia* tissue (Table III). According to him, fructose is the best carbon source, followed by mannose, galactose, glucose, etc., and lastly by lactose. The remarkable efficiency of fructose for *Sequoia* tissues explains Ball's finding that sucrose partially inverted by autoclaving allowed better growth than intact sucrose (5).

One of the most interesting peculiarities observed by Hildebrandt &

Riker (133, 134) in the course of their experiments, is the possibility of utilization of pectins, dextrins, and even starch. This implies the secretion by the tissues of specific hydrolases capable of transforming the given polysaccharide into directly utilizable sugars. The existence of such a secretion was demonstrated by Nickell & Burkholder (218) in cultures of sorrel virus tumors. These tissues can utilize various sugars, such as glucose, sucrose, fructose, maltose, raffinose, etc., (Table III) and, curiously enough, could be cultivated in media containing soluble starch. This utilization of starch has been studied in detail by Brakke & Nickell (28 to 30). Having placed tissue colonies on soluble starch, they found that the starch was slowly hydrolyzed, the nutrient solution becoming enriched in amylase. It is an active secretion and not a simple diffusion from damaged or crushed cells.

Brakke and Nickell have established, on the other hand, that isolated sorrel roots are incapable of utilizing starch. But certain crown gall tissues such as those of *N. tabacum* or *V. rosea* grow appreciably on media containing starch as the only carbohydrate. However, they do not utilize it as efficiently as the virus tumor tissue of sorrel, and, concomitantly, produce less amylase.

Other examples of starch consumption by tissue cultures are known. The most recent is that pointed out by Straus & La Rue in the course of their investigations on corn endosperm tissue culture (281). These tissues are capable of using the same carbohydrates as dicoteledoneous cells, i.e., sucrose, glucose, fructose, and maltose (Table III). But, just as the virus tumor tissues of sorrel, they can be cultivated on starch as the sole carbon source. We should finally note a very curious result obtained by Slabecka-Szweykowska (272). The enzymatic hydrolysate from starch constitutes the most efficient carbon source for vine tissues.

The investigators have not limited themselves to the establishment of a list of carbohydrates capable of acting as carbon sources, but they have also tried to determine the most favorable concentrations. Gautheret was the first to do such work (79), using carrot tissue. He showed that the efficiency of sucrose varied considerably with concentration. Its effect is first shown in concentrations of the order of 0.01 per cent but it is too weak for lasting proliferation. Optimal growth is attained with 3 per cent. Above this, growth diminishes but no toxic effects can be observed. Passage of a colony from a diluted to a concentrated medium has no effect, but the opposite transfer causes more or less generalized necrosis.

Similar experiments made by Hildebrandt & Riker (134) on tumor tissues also showed well-marked optima in the range of 2 to 4 per cent. Nickell (216, 217), working with sorrel virus tumor tissue, reached similar conclusions. In general, there seems to be no relation between the optimum level and the molecular weight of the sugar. The optimum corresponds frequently to the same concentration by weight of glucose or sucrose. What matters in growth, then, is not the amount of metabolizable sugar but rather the osmotic pressure of the solution. Nevertheless, in the case of *Sequoia* tissue cultures, the use of equimolar concentrations of glucose and sucrose furnished equivalent

TABLE III

CARBOHYDRATES UTILIZABLE BY PLANT TISSUE CULTURES*

Nature of carbohydrates	Normal carrot tissue [Gautheret (79)]	Crown Gall Tissues				Tissues of spontaneous tumors of the hybrid *Nicotiana langsdorfii* ×*N. glauca* [Hildebrandt & Riker (133, 134)]	Tissues of tumors induced on sorrel by the virus *Aureogenus magnivena* [Nickell & Burkholder (218)]	Corn endosperm tissues [Straus & LaRue (281)]	Tissues of *Sequoia sempervirens* (Ball, Pers. comm.)	Tissues of Sunflower [Henderson (129)]
		Tagetes erecta [Hildebrandt & Riker (133, 134)]	*Chrisanthemum frutescens* [Hildebrandt & Riker (133, 134)]	*Vinca rosea* [Hildebrandt & Riker (133, 134)]	*Helianthus annus* [Hildebrandt & Riker (133, 134)]					
HEXOSES										
Glucose	xxxx	xxxx	xxxxx	xxxxx	xxxxx	xxxx	xxxxx	xxxx	xxxx	xxxx
Fructose	xxx	xx	xxx	xxxx	xxxxx	xxx	xxxx	xxxx	xxxxx	xxx
Galactose	xxx	o	o	xxxxx	o	o	o	o	xxxx	–
Mannose	xx	xx	o	x	o	xx	–	–	xxxx	–
Sorbose	–	–	–	–	–	–	o	–	–	–
PENTOSES AND DERIVATIVES										
Arabinose	o	o	o	o	o	o	o	o	–	–
Xylose	o	o	o	o	o	o	o	–	–	–
Rhamnose	–	o	o	o	o	o	o	o	–	–
DISACCHARIDES										
Sucrose	xxxxx	xxxxx	xxxx	xxx	xxx	xxxxx	xxxx	xxxxx	xxxx	xxxxx
Maltose	xxxx	xx	xxx	xxx	x	xxxxx	xxx	xxx	–	–
Lactose	xx	o	o	xx	x	x	o	o	xxx	–
Cellobiose	–	xxx	x	xx	x	xxxx	xxx	–	–	–
Melibiose	–	–	–	–	–	–	xxx	–	–	–
TRISACCHARIDES										
Raffinose	xxxx	o	xx	xxx	x	xx	xxx	–	xxxx	–
POLYSACCHARIDES AND DERIVATES										
Soluble starch	o	x	o	xx	o	xx	xxx	xx	–	–
Glycogen	T	–	–	–	–	–	–	–	–	–
Inulin	o	o	o	o	o	o	–	–	–	–
Dextrin	o	x	x	xx	o	xx	–	–	–	–
Pectin	–	xx	x	xx	x	xxx	–	–	–	–

* For each tissue tested the nutritive value of a given compound is given the relative number of crosses. o means that the compound is only insignificantly assimilated and that practically it cannot serve as a carbon source. T corresponds to toxic substance; – means that the compound was not tested.

results [Ball (6)]. Finally, Straus & La Rue have observed (281) that the optimum concentration is not the same on a fresh weight basis as for dry weight. The optimum concentration of sucrose is thus 2 per cent on the basis of fresh weight, but 8 per cent if one measures dry weight.

Alcohols.—It was natural to investigate whether sugars could be replaced by certain polyalcohols closely related to them. Since 1948, Gautheret (90) undertook the cultivation of normal carrot tissue on glycerol as a carbon source. In the dark, growth was somewhat better than in the controls but necrosis occurred by the third or fourth passage. In light, the explants formed chlorophyll and under these conditions good growth was obtained even after 4 or 5 passages and also if the cultures were returned to darkness. It is thus possible to cultivate carrot tissue indefinitely on glycerol as the sole carbon source.

In 1949, Hildebrandt & Riker (133) attempted the cultivation of tumor tissue on media containing several alcohols: methanol, ethanol, propanol, butanol, glycerol, mannitol, etc. Only glycerol sustained appreciable growth which, however, was clearly inferior to that obtained with sugars. By using alcohols in conjunction with sucrose they established that certain alcohols such as phloridzin, propanol, and butanol were toxic; others such as mannitol are indifferent, while methanol, dulcitol, erythrol, and glycerol at low concentrations (0.5 per cent) induce added growth. Nickell & Burkholder (218) also tested individual alcohols but not in conjunction with sugars. The alcohols used (sorbitol, inositol, and mannitol) did not maintain appreciable growth, with the exception of glycerol.

In general, then, glycerol is the only polyalcohol having appreciable nutritive value. Corn endosperm tissues, however, are unable to utilize it, according to Straus & La Rue (281).

Acids.—In the course of his experiments on the replacement of sugars Gautheret (unpublished) tried to cultivate normal carrot tissue on media containing organic acids, particularly, oxalic, tartaric, and pyruvic acids. All of them were toxic even at concentrations of 0.2 per cent. This is surprising for pyruvic acid, since it is a normal cell constituent participating in intermediary metabolism. Hildebrandt & Riker (133, 134) tried sixteen different acids. They established that, in general, their nutritive value was insignificant. Some, such as succinic, glutaric, and fumaric gave an amount of growth which never exceeded 15 per cent of that obtained with sucrose. Others, such as gluconic and malic appeared to be nonutilizable. They also tried sucrose in conjunction with a 0.5 per cent concentration of these acids. The calcium salts of succinic, malic and gluconic acids gave added growth; fumaric, tartaric, or stearic were indifferent or slightly inhibitory; and formic, acetic, oxalic acids, etc., were toxic.

Nickell & Burkholder (218) carried out less discouraging studies with sorrel virus tumor tissue. They found that some acids such as fumaric, succinic, citric, and aspartic were utilizable as carbon sources with a relative efficiency of 25 to 50 per cent compared to sucrose. Others, like lactic and pyruvic were always toxic.

Absorption and transformation of carbohydrates.—It is known that plant cells can transform absorbed carbohydrates. The work of Colin and his school has furnished numerous examples of these transformations (49). But the methods of classical physiology led sometimes to confusion; the changes in carbohydrate content that they reveal can be due to true transformations or to simple migratory processes which are difficult to distinguish. Willam has pointed out an example of flagrant error (304). The method of tissue culture which allows cells to be supplied a perfectly defined medium and thus rules out migration, is a choice method for the study of the absorption of sugars and their transformations within tissues.

Starting in 1942, the Italian worker Cova (50) performed preliminary experiments on isolated carrot root fragments. These fragments contained initially appreciable amounts of mono- and oligosaccharides. He found that in spite of being cultivated in a medium having 2 per cent glucose the explants consumed their carbohydrate supply. This consumption proceeded faster in winter than in summer and involved mostly the reducing sugars; the nonreducing sugars could even increase during the winter. Cova established that tissue newly formed during culture contained more carbohydrates than the preexistent tissue; it could even be richer than the primitive explant if 5 per cent sugar was used.

This first work merely sketched the study of the absorption and transformation of carbohydrates by tissue colonies. It was largely extended by Goris, who carried out a long series of experiments on organ fragments cultivated in various media. Working first with sugar-free media he confirmed Cova's results concerning the influence of season on the rate of carbohydrate consumption in carrot fragments (109 to 112). He also established that proliferation varied concurrently with carbohydrate consumption indicating that consumption of sugars was a simple consequence of the formation of new cells. This opinion was strengthened by certain experiments (to be examined later) with auxin (115).

These preliminary investigations were followed by others designed to study the penetration of a given sugar, glucose, fructose, or sucrose, and its transformation within the tissues. Goris irrefutably demonstrated that each one of these sugars can give rise to the other two (115). One of these transformations, the interconversion of glucose and fructose, deserves special mention. The existence of such interconversion was suspected for a long time, but only the method of *in vitro* tissue culture permitted its demonstration. Goris also found that the transformation fructose→glucose occurs far more easily than the opposite transformation. Similarly, fructose produces more sucrose than an equivalent amount of glucose, at any rate in carrot tissue subjected to numerous transfers.

Finally, for the same species, the rate of these transformations varies according to the tissues. Thus, in vascular parenchyma of carrot sucrose synthesis is more active than in phloem tissue (115). One sees that the method of tissue culture has allowed Goris to make a precise analysis of carbohydrate transformations.

Other authors, too, have taken up this problem. Ball (6) has studied the carbohydrate transformations taking place in normal tissue of *Sequoia sempervirens* cultivated in media containing one of the following sugars: glucose, fructose, sucrose, mannose, glactose, raffinose, or lactose. He did not perform quantitative measurements but characterized chromatographically the sugars contained in the explants and in the media. Tissues removed from the normal plant contain a mixture of sucrose, glucose, and fructose; this mixture remains unchanged if the colonies are cultivated in any one of the three sugars. If one uses mannose or lactose as a carbon source, then the new sugar is simply added to the original mixture. But if galactose is used, one finds raffinose in the colonies. Reciprocally, media containing raffinose give rise to galactose. Ball has also found that at the end of the culture the nutrient medium has the same sugars as are found in the colonies. This undoubtedly results from exosmosis of the sugars from the cells. Complementary work on crown gall tissue from *V. rosea* has furnished the same general results.

Growth Stimulants

Certain normal tissues, for example, the cambial tissue of carrot or bramble and numerous tumor tissues such as those of crown gall, can proliferate on a medium containing mineral salts and a sugar exclusively. With others, one is forced to use several organic substances which stimulate cell proliferation. These substances are either definite compounds, generally synthetic, or natural products of ill-defined composition.

The existence of substances capable of causing cell multiplication was well known before plant tissues were cultured. In 1929, Hammet (122) had shown that cysteine and other sulfur compounds were capable of inducing meristematic proliferation. Some years later Snow (273) discovered that indoleacetic acid stimulates the operation of the phloem-xylem generating zones. Finally, Bonner (21), finding inspiration in White's work (288), demonstrated in 1937 that vitamin B_1 was necessary for the development of the root meristems of certain species.

These findings were naturally taken advantage of by the first investigators to attempt tissue cultures: Gautheret, Nobécourt, and White. Later, other stimulating substances were used such as auxins, vitamins, organic bases, or amino acids. Still later, after van Overbeek, Conklin & Blakeslee (232) had discovered that coconut milk favors the development of isolated embryos, the specialists of tissue culture employed this substance as well as other natural products such as extracts of seeds, fruits, yeast, etc. We shall consider first the stimulating properties of well-defined substances and then those of the ill-defined natural products.

Auxin production by tissue cultures.—The use of auxins has had a decisive role in normal tissue culture, since the tissues of most dicots only proliferate in the presence of such substances. The first known example was that of Jerusalem artichoke tissue. Gautheret showed that if one removes

tuber fragments of certain varieties in the winter, they will not grow when placed in an auxin-free medium (81). Intense proliferation is obtained with 10^{-8} to 10^{-6} gm. per ml. of indoleacetic acid. On the other hand, if one removes the explants in the fall from young rhizomes, or in the spring from germinating tubers, they develop appreciably even if the medium is devoid of auxin [Gautheret (82)]. The tissues of some varieties are capable of proliferating in winter in auxin-free medium [Kulescha & Gautheret (169)]. With all tissues, however, the newly-formed tissue cannot be successfully transferred into a medium from which auxin is absent.

Kulescha has shown further that the fluctuations in the capacity for spontaneous proliferation of Jerusalem artichoke tissues bears a direct relation to the auxin content (157, 159); thus auxin decreases in winter and increases on resumption of growth. The isolated fragments slowly deplete the low amount of auxin which they contain and after one month they have practically none [Kulescha (157)]. This wealth of facts indicates that isolated Jerusalem artichoke tissues are heterotrophic with regard to auxin; they do not synthesize the required amounts and therefore auxin has to be supplied externally.

Gautheret demonstrated that the same is true for tissues of *Scorzonera* (78) and Morel arrived at the identical conclusion as a result of his work on normal tissues of Virginia creeper, hawthorn, and some other dicots (193). Czosnowski (51) recently showed that dahlia tissue behaves exactly the same as that of Jerusalem artichoke.

Those tissues capable of growing without an external addition of auxin are auxin-autotrophic, i.e., they produce the necessary amounts and it is useless to supply it to them [Kulescha (159); Kulescha & Gautheret (164); Henderson & Bonner (130)]. This is true of normal tissues of bramble and willow, of crown gall tissue of *Scorzonera*, Jerusalem artichoke, etc., of the genetic tumor tissue of the *N. langsdorfii*×*N. glauca* hybrid [White (290)] and finally of the tissues from tumors produced on sorrel by the virus *A. magnivena* [Black (18)]. In general, tumor tissue can be cultivated without auxin, the only known exception being the tissues from the galls produced on *Salvia pomifera* by the insect *Aulax* sp. Démétriades (54) has shown that the latter tissues will not develop in the absence of auxin. This may be true of tissues induced by other coecedia.

The fact that tumor tissue produces more auxin than normal tissues has attracted the attention of many workers and various explanations have been advanced. Henderson & Bonner (130) believe that the low auxin concentration of normal tissues is due to an inhibitor of the enzyme which transforms tryptophan into indoleacetic acid. This opinion is not irreproachable since Kulescha has found that normal Jerusalem artichoke or *Scorzonera* tissues can make auxin from tryptophan as easily as crown gall tissues (158, 159).

Bitancourt (15) has suggested a completely different explanation. According to him, the abundance of auxin in tumors would result, not from increased production, but rather from a weaker destruction. In other words,

normal tissue would destroy auxin faster than tumor tissue. Platt has undertaken to use this hypothesis in his methodological studies on the inactivation of auxin by enzymes specific to tumor tissues (242). He has considered particularly the system peroxide-peroxidase to which a role is attributed in indoleacetic acid destruction. His work has not yielded conclusive results. It would appear that the peroxidases studied by Platt are without effect in the oxidation of indoleacetic acid and, further, that auxin inactivation by this type of enzymatic system does not play an essential role in the limitation of tissue growth.

Thus, the investigations done on auxin formation and destruction have not revealed the mechanism whereby tumor tissues contain high auxin levels. These investigations postulate that the auxin from normal and tumor tissue is the same, i.e., indoleacetic acid or related compounds. Bitancourt *et al.*, have submitted this postulate to examination by means of chromatographic analyses (16, 17). They have found that the auxins from both types of tissue are, in general, the same. But other analyses have yielded different results. Kulescha has shown that the auxin extraction differs according to the tissue (161). Extraction is instantaneous from tumor tissues but occurs progressively in the case of normal tissues. These results could be explained either by postulating different auxins, contrary to Bitancourt's ideas, or by adopting Henderson & Bonner's opinion that normal tissues contain an inhibitor of the enzyme converting tryptophan into indoleacetic acid (130). One must remember that the chemistry of natural auxins is fragmentary. Thus, by treating Jerusalem artichoke tissues with indoleacetic acid or 2,4-dichlorophenoxyacetic acid, Schoen & Morel (261) found that these substances did not accumulate in the explants but that other auxins of unknown nature could be extracted from them.

Thus, the most recent investigations again raise the problem of auxin in normal and tumor tissues. At present, it is not even possible to explain the spontaneous proliferation of tumor and certain normal tissues by an enhanced power of auxin synthesis. The only irrefutable result is that while some tissues can be cultured without auxin, others require a substance of this type.

Effect of auxins upon tumor tissues.—The fact that tumor tissues are auxin-autotrophic has not discouraged studies on the effects of auxins on their proliferation. It has been ascertained that, as a whole, auxins either do not stimulate[3] or have an insignificant effect on their increase in weight [de Ropp (254); Gautheret (88); Hildebrandt & Riker (132); Morel (193); Nickell (209)]. This action, when manifested, cannot be attributed *ipso facto* to increased proliferation, as auxin also causes cell enlargement. The histological findings of Struckmeyer, Hildebrandt & Riker (282) have shown that auxins increase cell size in tissue colonies of sunflower crown gall.

[3] It must be remembered, however, that the gall tissue from *S. pomifera* is very responsive to auxin stimulation [Démétriades (54)].

The reactivity of *Scorzonera* crown gall tissue to auxins depends to a certain extent on the physiological state (Paris and Duhamet, personal communication). Explants taken from "anergized" (habituated, see later) colonies show an appreciable sensitivity, at least if the cultivation is made in Gautheret's medium plus 0.4 gm. KCl per liter. This medium allows particularly rapid proliferation and that is probably why auxin is effective. These results may indicate that tumor tissues really show an appreciable sensitivity toward auxin stimulation and that such sensitivity is masked under certain conditions.

It is nonetheless true that in practice tumor tissues are cultivated in auxin-free media. We must note, however, that Nickell uses 2,4-dichlorophenoxyacetic acid for *Datura* crown gall tissue and *para*-chlorophenoxyacetic acid for tumor tissue of *M. officinalis* (see Table IV), but in conjunction with coconut milk or yeast extract, respectively, so that the exact role of these auxins remains unknown.

Effect of auxins upon normal tissues.—Auxins are used in the cultivation of normal tissues (Table IV). In some cases (7 species) indoleacetic acid (10^{-8} to 10^{-6} gm. per ml.) is used. In others (33 species) naphthaleneacetic acid (5×10^{-8} to 10^{-6} gm. per ml.) is employed. *Para*-chlorophenoxyacetic acid (6×10^{-7} gm. per ml.) is used for normal tissues of *M. officinalis* and *Vigna sinensis*. This last auxin has been introduced by Jagendorf & Naylor (148), who found that it caused the formation of tumor-like structures on cabbage roots. Later, Jagendorf & Bonner established that such tumors could be used as a starting point for tissue culture (147). Finally, 2,4-dichlorophenoxyacetic acid in concentrations 10^{-8} to 6×10^{-6} gm. per ml. is utilized in the culture of normal tissues of six species.

It is known that the stimulating action of auxins on cellular multiplication is exerted mainly on cambial tissue and parenchymatic tissue derived from it [Gautheret (94)]. That explains their powerful effect on normal tissues of dicots and Gymnosperms. Their effect on certain monocot tissues such as those of corn and *Amorphophallus* [Morel (194)] or that of *Agave* (Nickell, pers. comm.) is not well understood. Their role in these tissues must be slight as they are used in conjunction with coconut milk which causes a powerful and diffuse proliferation of parenchyma.

For a given species, the effect of auxin depends on the concentration. Thus, a low concentration of indoleacetic acid produces proliferation in carrot tissue [Gautheret (81)]; at a concentration of the order of 10^{-6} gm. per ml. it stimulates rhizogenesis and at 10^{-5} or 10^{-4} gm. per ml. produces generalized swelling of the cells. This swelling may be confusing as it leads to a considerable increase in size of the explants without any proliferation taking place. Thus, in order to obtain durable cultures, the correct concentrations which selectively cause cell division must be employed. These concentrations become more and more effective as cultures are prolonged since they cause a progressive dedifferentiation, while higher concentrations bring about a more or less complete differentiation of the explants which finally

lose their efficiency. Auxin, therefore, gives rise to a phenomenon of sensitization [Gautheret (81)] which, in one case or the other, favors or opposes the establishment of the culture. The pernicious effects of high auxin levels have caused some vexation. An examination of Figures 5, 8, and 14 of the paper published in 1947 in the *American Journal of Botany* by de Ropp (254) convincingly shows that high auxin levels produce giant cells incapable of multiplication. In his researches on sunflower tissue culture Henderson, too, (129) obtained evidence that at high concentrations auxin does not allow lasting tissue growth.

The variability of tissue reactions in response to the auxin level is observed in most tissues but the concentrations corresponding to each response differ with the species. Therefore, the levels used to obtain lasting cultures vary from one tissue to the other. With vine tissues, which produce giant cells with ease under the influence of auxins, one must be cautious (Table IV). The same is true with tobacco tissue. Jablonski & Skoog (139) found that indoleacetic acid does not produce proliferation but rather cell enlargement in tobacco pith fragments. It is not, however, a simple process of elongation, since Naylor, Sander & Skoog (201) observed that the nucleus divides but without the subsequent appearance of walls. The production of the latter can occur only if the explants include some conducting tissue which appears to supply some specific factor capable of acting in conjunction with indoleacetic acid.

The tissues of *Syringa*, on the contrary, produce practically no giant cells, so higher levels can be used. It must be noted that certain auxins appear to have a callus-inducing action only. Thus, *para*-chlorophenoxyacetic acid causes callus formation in cabbage roots [Jagendorf & Naylor (148)]; these calluses can be pricked successfully into a medium containing the same auxin [Jagendorf & Bonner (147)]. If the auxin is withheld, the culture is arrested and the colonies become transformed into masses of roots. This is an unexpected result as auxins ordinarily favor root formation.

Finally, tissues from different varieties of the same species may show different auxin requirements. Gautheret was able to cultivate normal tissues from the Muscade variety of carrot without auxin (95) while Nobécourt (223) and Levine (176) using unknown varieties were forced to use high auxin concentrations (10^{-6} to 5×10^{-6} gm. per ml.). On the other hand, de Ropp *et al.* (259) were unable to culture tissue from the Chantenay variety in auxin-containing media. They had to resort to the use of coconut milk, the stimulating power of which is superior to that of auxins. Steward and Caplin (personal communication) have also used coconut milk for cultivating tissue from an unknown carrot variety. Wiggans (301), however, obtained indefinite tissue culture with the Chantenay variety by using indoleacetic acid. Virginia creeper tissues show the same irregularity as those of carrot. Morel could cultivate them only by using naphthaleneacetic acid (193), while Bertossi in Italy (personal communication) isolated a strain without resorting to any division-stimulating substance.

Numerous tissues can be cultivated with an auxin as the sole stimulating substance, for example those of *Althea thuringiaca*, *Antirrhinum majus*, *Malva silvestris*, *Pyrus communis*, *Parthenocissus tricuspidata* [Morel (193)], *N. tabacum* (Hildebrandt), *Scorzonera hispanica*, *Helianthus tuberosus* [Gautheret (80)], *S. sempervirens* [Ball (3)], etc. In most of these tissues the substance acts only as a cell division stimulant. But in others it also acts as a bud-inhibitor. Thus, Gautheret used naphthaleneacetic acid for the cultivation of endive tissues; this auxin stimulates proliferation and at the same time inhibits the bud initiation which would interfere with the culture. Some tissues need, in addition to auxin, other growth factors such as vitamins, coconut milk, peptone, etc., the effects of which will be examined later. Morel (193), Steward & Caplin (274), Nickell (personal communication), Henderson, Durrell & Bonner (131), Kandler (149), Jacquiot (personal communication), Morel & Wetmore (197), and Loewenberg & Skoog (183) have given numerous examples of cultures needing auxin plus some other type of stimulating substance.

Applications of tissue culture to the study of the physiological properties of auxins.—The tests usually employed to determine auxin activity, such as the pea test or the *Avena* test, are based on cell elongation and thus deal with but one of the effects of auxins. The use of isolated roots is not more specific as this test deals with responses which result from a combination of cell enlargement and cell division, two processes which are often difficult to separate. The interpretation is still more complex for tests which involve epinasty or rhizogenesis. A very short time ago, Besset (14) perfected a truly specific test for cell division substances which can be used in the study of the inductive and formative effects of auxins. If the substance tested is a factor involved in cell division, a multiplication of cambial cells is observed.

The utilization of tissues sensitive to cell division substances, such as those of Jerusalem artichoke or carrot, gives an even more direct method for the assay of the stimulating properties of natural and synthetic auxins. Bouriquet *et al.*, studied in this way the activity of α-naphthylvinylacetic acid (25), N-methyl-*N*-phenylglycine (26) and of several derivatives of phenoxyacetic and naphthoxyacetic acids (24), used singly or in conjunction with structural analogues. Raoul & Gautheret also used tissue cultures to compare the activities of 2,4-dichlorophenoxyacetic acid, naphthoxyacetic acid, and indoleacetic acid (243) and to reveal the synergy between indoleacrylic acid and tryptophan (244). Longchamp & Pacheco used Jerusalem artichoke tissue cultures (184) to secure evidence of the inductive and formative properties of 2,4-dibromophenylglycine. And the same test enabled Bertossi to show that although diphenylacetic acid is an antagonist of indoleacetic acid (10) it has, nevertheless, the properties of a cell division factor (9). It is thus seen that tissue culture can furnish invaluable tests for the study of the stimulative and formative activities of auxins.

Auxins exert an important effect on the proliferation of cambium-type cells. They have been responsible for the most important successes obtained

TABLE IV

Use of Auxins in Plant Tissue Cultures

This table shows the conditions (nature and concentrations) under which auxins are used for the cultivation of different tissue stocks. They are always normal tissues, except for the rare cases noted in the column "Remarks"

Nature of the auxin	Concentration (Gm. per ml.)	Species	Name of author who cultivated the tissue	Remarks
Indoleacetic acid	2.5 to 5×10^{-6}	*Daucus carota* L.	Nobecourt (223)	The growth-factor requirements depend on the variety: Gautheret (95) cultivated tissue from the Muscade variety without auxin. However, de Ropp (259) and later Caplin and Steward (Pers. comm.) could not obtain growth of tissue from the Chantenay variety even with auxin, they had to resort to coconut milk.
	10^{-6} to 5×10^{-6}	*Daucus carota* L.	Levine (174)	
	10^{-6} to 10^{-7}	*Helianthus annuus* L.	Henderson, Durrell & Bonner (131)	
	10^{-6}	*Helianthus annuus* L.	Kandler (149)	
	10^{-6}	*Linum usitatissimum* L.	Ledingham	Personal communication
	10^{-7}	*Solanum tuberosum* L.	Bertossi	Personal communication
	10^{-6}	*Robinia pseudacacia* L.	Jacquiot	Personal communication
	10^{-6}	*Ginkgo biloba* L.	Tulecke (285)	
	10^{-6}	*Sequoia sempervirens Endl.*	Ball (3)	
Naphthaleneacetic acid	5×10^{-8}	*Vitis vinifera* L.	Morel (193)	
		Parthenocissus tricuspidata (Sieb. and Zucc.) Planch	Morel (193)	
	10^{-7}	*Brassica oleracea* L. (var. *Napobrassica*)	Gautheret (84, 104)	
	10^{-7}	*Nicotiana tabacum* L.	Hildebrandt	Personal communication
	10^{-7}	*Cissus incisa* (Torr. and Gr.) Desmoul	Morel (193)	
	10^{-7}	*Parthenocissus quinquefolia* L. Planch.	Morel (193)	
	10^{-7}	*Gladiolus* sp.	Morel (195)	
	10^{-7}	*Lycopodium cernuum* L.	Wetmore & Morel (287)	
	10^{-7}	*Pinus strobus* L.	Loewenberg & Skoog (183)	
	2×10^{-7}	*Helianthus tuberosus* L.	Gautheret (84)	
	3×10^{-7}	*Cichorium intybus* L. (var. Witloof)	Gautheret (84)	
	3×10^{-7}	*Scorzonera hispanica* L.	Gautheret (84)	
	3×10^{-7}	*Hyoscyamus niger* L.	Gautheret & Telle (284)	
	3×10^{-7}	*Althea thuringiaca* L.	Morel (193)	
	3×10^{-7}	*Malva silvestris* L.	Morel (193)	
	5×10^{-7}	*Salix caprea* L.	Gautheret (89)	
	5×10^{-7}	*Althea officinalis* L.	Morel (193)	

TABLE IV (*Continued*)

Nature of the Auxin	Concentration (Gm. per ml.)	Species	Name of author who cultivated the tissue	Remarks
	5×10^{-7}	*Antirrhinum majus* L.	Morel (193)	
	5×10^{-7}	*Malva crispa*	Morel (193)	
	5×10^{-7}	*Crataegus monogyna* Sweet	Morel (193)	
	5×10^{-7}	*Rubus reflexus* Ker.	Morel (193)	
	5×10^{-7}	*Amorphophallus campanulatus* Blume	Morel (194)	Personal communication
	5×10^{-7}	*Amorphophallus rivieri* Dur.	Morel & Wetmore (197)	
	5×10^{-7}	*Bellevalia romana* Rch.	Morel	Personal communication
	5×10^{-7}	*Lilium formosanum* Wallace	Morel	Personal communication
	5×10^{-7}	*Sauromatum guttatum* Schott.	Morel (196)	
	5×10^{-7}	*Zea mays* L.	Morel	Personal communication
	5×10^{-7}	*Ginkgo biloba* L.	Morel	Personal communication
	5×10^{-7}	*Pinus strobus* L.	Morel	Personal communication
	10^{-6}	*Pyrus communis* L. (var. Conference)	Morel (193)	
	10^{-6}	*Syringa vulgaris* L.	Morel (193)	
	10^{-6}	*Ulmus campestris* L.	Jacquiot	Personal communication
	10^{-6}	*Picea abies* Karst.	Loewenberg & Skoog (183)	
	10^{-6}	*Pinus banksiana* Lamb.	Loewenberg & Skoog (183)	
Para-chlorophenoxyacetic acid	6×10^{-7}	*Melilotus officinalis* Lamb.	Nickell	Personal communication
	6×10^{-7}	*Melilotus officinalis* Lamb.	Nickell	Personal communication Tumor tissue (Crown gall)
	6×10^{-7}	*Melilotus officinalis* Lamb.	Nickell	Personal communication Tumor caused by the virus *Aureogenus magnivena*
	6×10^{-7}	*Vigna sinensis* Endl.	Nickell	Personal communication
	1.5×10^{-7}	*Brassica oleracea*	Jagendorf & Bonner (147)	
2,4-Dichlorophenoxyacetic acid	10^{-8}	*Persea americana* Mill.	Morel	Personal communication
	6×10^{-7}	*Datura stramonium* L.	Nickell	Personal communication Tumor tissue (Crown gall)
	6×10^{-7}	*Rumex acetosa* L.	Nickell	Personal communication
	6×10^{-7}	*Persea americana* Mill.	Nickell	Personal communication
	6×10^{-7}	*Agave toumeyana* Trel.	Nickell	Personal communication
	6×10^{-6}	*Solanum tuberosum* L.	Steward & Caplin	Personal communication

with tissue cultures. It must be remembered, however, that auxins should be used with discretion since the optimum concentrations lie close to the toxic levels.

Vitamins.—The first vitamin to be used by tissue culture specialists was

vitamin B_1. The fact that it is frequently needed for the cultivation of isolated roots, suggested to White (292), Gautheret (76), and Nobécourt (223) its systematic use for tissue colonies. But in 1940 Nobécourt found that carrot tissues elaborate it in appreciable amounts (224) and he deemed its use unnecessary in tissue cultures. Hildebrandt, Riker & Duggar (136) arrived at a similar conclusion for sunflower and tobacco tumor tissue. Gautheret confirmed these results for normal carrot tissue and even showed, as was mentioned before, that tissue from the Muscade variety grew independently of any growth factor (95). Working on the same material, Czosnowski (53) determined the amount of vitamin B_1 in tissue colonies cultivated in thamin-free media, and found it to be of the order of 5×10^{-8} gm. per ml. Similar analysis made by Paris (233) gave the same value. Czosnowski and Paris further established that addition of vitamin B_1 had no effect on tissue growth.

This is however, not always the case. *Scorzonera* crown gall tissue, as well as that of carrot, can thrive without vitamin B_1 since they synthesize appreciable quantities, but the presence of this vitamin in the medium (at about 5×10^{-8} gm. per ml.) intensifies proliferation [Paris (233)]. This vitamin exerts the same influence on vine tissues which have been subject to "auxin anergy" (see later). Czosnowski has shown that by pricking colonies to vitamin-free media, proliferation decreases after nine passages and then increases to the normal level if the vitamin is added. An extreme case was discovered by Nickell (213) in the tumor tissues of sorrel caused by the virus *A. magnivena*. For them, thiamin is the sole but essential growth factor needed.

Another vitamin of the B group, pantothenic acid, plays an important role in the growth of certain tissues. In 1946, Morel (192) established that a low level (5×10^{-7} gm. per ml.) of this substance favors callus production by hawthorn stem fragments. Its action, though less powerful is synergistic with that of naphthaleneacetic acid. Gautheret (89, 284) found that pantothenic acid stimulates tissue proliferation in willow and black henbane. Cznosnowski, on the contrary, could observe no effects of this vitamin on carrot, vine and Virginia creeper tissues, which synthesize it in rather appreciable amounts (of the order of 10^{-6} gm. per ml.).

Meso-inositol has been used in some experiments. The first were those of Jacquiot (143) who established that relatively high levels (2×10^{-4} to 10^{-3} gm. per ml.) of this vitamin favor bud formation by elm cambial tissue and retards necrosis without intensifying, however, the proliferation of colonies.

Among other vitamins tested, *p*-aminobenzoic acid should be noted. It stimulates proliferation of normal Jerusalem artichoke tissue and acts synergistically with indoleacetic acid [de Capite (42)]. Czosnowski, however, showed that it has no activity on several other tissues (53).

Interesting but contradictory data have been obtained with folic acid. De Ropp (258) considers this vitamin to be inactive, an opinion which Czosnowski (53) justifies by showing that tissues elaborate appreciable quantities (1 to 6×10^{-8} gm. per ml.). Other investigations by Capozzi (47) show that

in the dark, folic acid slows tissue proliferation, while enhancing it in light. According to him, this enhancement results indirectly. In light, folic acid hydrolyzes to yield several degradation products, among which is *p*-aminobenzoic acid, a stimulating substance [de Capite (42)].

Vitamin K_5 has been studied by Duplessy-Graillot (66, 67). Used alone, it inhibits, but in conjunction with 2,4-dichlorophenoxyacetic acid, its antagonistic effect results in an alleviation of the depressive effect of high levels of the auxin.

Hildebrandt, Riker & Duggar (136) consider pyridoxin to be slightly stimulatory while Czosnowski (53) has observed no activity, which he explains by the fact that tissues synthesize it in large amounts (10^{-7} to 10^{-6} gm. per ml.). According to him, tissue colonies are also autotrophic with regard to riboflavin, nicotinic acid, and biotin.

For practical purposes, vitamins are used in mixtures. For example, in investigations on the culture of normal sunflower tissue Henderson, Durrell & Bonner (131) and then Jagendorf & Bonner (147) used a mixture of ten vitamins (Table V), perfected on the basis of work done on *Neurospora* genetics. This mixture is effective, but whether all the constituents are really necessary or even useful is unknown.

Other mixtures used by Morel (193), Gautheret (89), Morel & Wetmore (198) and Jacquiot [personal communication, and (141)] also have an empirical composition. The formula proposed by Paris & Duhamet (234), on the other hand, is based on trials undertaken with the aim of obtaining a synthetic medium with the same stimulatory and formative properties possessed by coconut milk. The latter contains vitamins and amino acids.

TABLE V

CONSTITUTION OF SOME VITAMIN MIXTURES USED IN PLANT TISSUE CULTURES
(Concentrations in gm. per ml.)

Vitamins	Willow Gautheret (89)	Pteridophytes Morel & Wetmore (198)	Various dicots Morel (193)	Normal sunflower tissues Henderson Durrell & Bonner (131)	Tissues from trees Jacquiot (141)	*Scorzonera* crown gall Paris & Duhamet (234)
Vitamin B_1	10^{-6}	10^{-6}	10^{-6}	10^{-7}	10^{-6}	5×10^{-9}
Ca pantothenate	10^{-6}	10^{-6}	10^{-6}	8×10^{-7}	5×10^{-7}	5.2×10^{-8}
Biotin	10^{-8}	10^{-8}	10^{-8}	5×10^{-9}	10^{-7}	2×10^{-9}
Meso-inositol	—	10^{-4}	10^{-4}	5×10^{-7}	5×10^{-4}	—
Riboflavin	—	—	—	5×10^{-8}	10^{-7}	10^{-6}
Pyridoxin	—	10^{-6}	—	10^{-7}	—	10^{-6}
Para-aminobenzoic acid	—	—	—	5×10^{-8}	10^{-6}	—
Nicotinic acid or amide	—	10^{-6}	—	5×10^{-7}	10^{-6}	6.4×10^{-8}
Choline	—	—	—	10^{-7}	—	—
Folic acid	—	10^{-6}	—	10^{-7}	10^{-9}	3×10^{-10}

When these amino acids and these vitamins are used individually with *Scorzonera* crown gall tissue, the growth of the colonies is only slightly better than that obtained without stimulatory substances. But by combining both types of substances (seven vitamins and eleven amino acids) one obtains a proliferation comparable to that observed with coconut milk. The mixture, however, does not exactly replicate coconut milk, since with carrot tissue, the effect of the former is clearly less than that of the latter [Paris & Duhamet (235)]. Miss Paris has further revealed that a mixture of meso-inositol, thiamin, and biotin is active if combined with indoleacetic acid or coconut milk (233).

Ascorbic acid has been used sometimes not to stimulate colony growth, but to stop melanin formation which is toxic to the explants. Morel has used ascorbic acid in the cultivation of *Equisetum* and aconite's tissue (personal communication).

Amino acids.—The pioneers of tissue culture employed amino acids. For the cultivation of tumor tissue White used the glycine-containing medium which he perfected during his investigations on root culture (292). Nobécourt and Gautheret, prompted by Hammet's work (122) on the stimulatory properties of −SH radicals, added cysteine to their nutrient media (76, 223). After that, Nobécourt (226) and Gautheret (95) showed that cysteine was not necessary for the growth of carrot tissue, but according to Wiggans (301) it nevertheless stimulates their growth. Glycine, too, appears to play an unimportant role. Riker & Gutsche (247) could observe no stimulation due to it. Nickell & Burkholder (218) arrived at similar results, although they established the not surprising fact that glycine could moderate nitrogen deficiency. The extended series of experiments carried out by Riker & Gutsche on the nitrogen nutrition of sunflower crown gall tissue (247) have shown that numerous amino acids (histidine, leucine, valine, lysine, etc.) are indifferent at weak concentrations but become toxic as soon as the levels are increased. Three of them, however, alanine and aspartic and glutamic acids, behave peculiarly; they inhibit at medium concentrations but become inactive at lower or higher concentrations. These results are valid only if nitrate is not limiting. When nitrate is deficient, the nitrogen supplied by the amino acids complicates the course of the experiments. Nickell & Burkholder (218), working with sorrel virus tumor tissue, also found that amino acids lack stimulatory properties, with the exception of aspartic acid which permits a notable increase in colony proliferation, even in the presence of optimum nitrate concentration (208).

Tryptophan has produced contradictory results. Riker & Gutsche (247), as well as Nickell & Burkholder (218), have obtained no stimulation with this amino acid. Nobécourt, working on carrot tissue, arrived at a similar conclusion (225). But in 1949, Kulescha & Gautheret (165) discovered that it displays stimulatory and formative properties similar to those of auxins, i.e., it stimulates proliferation of normal tissues (carrot and Jerusalem artichoke) but has no effect on tumor tissues (*Scorzonera* crown gall). Its activity

is about 100 times less than that of indoleacetic acid, i.e., it causes the same reactions but at concentrations 100 times higher.

Tryptophan is a very labile substance which, by heating or simple contact with an agar medium, is converted into a substance capable of giving the *Avena* reaction, so that Kulescha and Gautheret were led to suspect their results. They improved their technique so as to preclude any alteration of tryptophan with the result that they still obtained stimulation. On the other hand, it was possible to show that Jerusalem artichoke tissue [Kulescha (158)], cultivated in the presence of tryptophan, produced auxin, there being a direct relation between auxin content and proliferation. The intensity of proliferation is the same as that which would be obtained with an auxin concentration in the medium equal to that found within the explants. It appears as if the stimulatory properties of tryptophan result from its intracellular conversion to indoleacetic acid. The results of Kulescha (159) confirm those obtained by Wildman, Ferri & Bonner (303) on the transformation of tryptophan to indoleacetic acid in spinach leaves which were not at all conclusive as the conversion could have occurred in their experiments through the agency of microorganisms. This objection does not apply to tissue cultures, where one is assured of sterility. Another product of tryptophan metabolism, kyneurenin, slightly stimulates proliferation of Jerusalem artichoke tissue [Kulescha & Gautheret (166)]. Another amino acid, arginine, though not stimulating the growth of tissue colonies, is not entirely inactive, as it favors the formation of new buds [Skoog & Tsui (271)].

Thus, it appears that amino acids taken singly only rarely possess stimulatory properties. Amino acid mixtures on the contrary, can be active. Paris & Duhamet (234) have shown that the mixture of eleven amino acids contained in coconut milk stimulates appreciably the proliferation of certain tissues. It may be that the activity of the mixture is due simply to the presence of aspartic acid but there is nothing against it being a phenomenon of synergy. Straus & La Rue (281), studying corn endosperm tissue culture, have confirmed the favorable effect of certain amino acid mixtures. Finally, Nétien & Beauchesne have observed the same for carrot and Jerusalem artichoke tissues (203).

Organic bases and nucleic acid derivatives.—Tissue proliferation implies cellular multiplication which, in turn, needs, according to all available evidence, the intervention of nucleic acids. It was thus natural to ask whether the constituents of these acids, especially the purine and pyrimidine bases, are capable of stimulating tissue growth.

The first studies of this kind were those of Skoog and his collaborators. They were done with adenine (267, 268, 270, 271) which Skoog discovered induced bud formation on tobacco stem fragments. One cannot say, however, that adenine is the specific caulogenic factor as it does not induce bud formation in those tissues which are incapable of producing them spontaneously. Its caulogenic effect is similar to the rhizogenic activity of auxins which are also without action on tissues which cannot by themselves give rise to root

meristems. This conclusion is corroborated by certain experiments of Jacquiot (143) which have revealed that in elm tissue, bud formation can also be influenced by meso-inositol, the intensity of bud formation being a function of the ratio of meso-inositol to adenine.

Skoog and his collaborators, Stone (280) and Miller (190), have established that other organic bases (guanine, hypoxanthine, and thiothymine) are equally capable of stimulating caulogenesis. Adenine manifests synergistic effects with guanine, thymine, and cytosine. Wiggans (301) obtained curious results in his cultures of carrot in media with adenine. Tissue growth was stimulated but bud formation was elicited only after the explants were transferred to adenine-free medium.

The stimulative and formative activity of adenine is far from general. Nickell, Greenfield & Burkholder (219) found that it inhibits the proliferation of the virus tumor tissues of sorrel. Henderson, Durrell & Bonner obtained equally discouraging results with sunflower tissue (131).

Other degradation products of nucleic acids have been considered by Nickell *et al.* in the course of their experiments with sorrel virus tumor tissues (219). Ribonucleic acid hydrolyzate is stimulatory, while that of desoxyribonucleic acid is slightly inhibitory. This difference may be due to the presence in the colonies of a ribonucleic acid-rich virus. Both acids differ in the nature of their organic bases. Uracil, contained in ribonucleic acid, is stimulatory, while the guanine of desoxyribonucleic acid is inactive. The nucleotides behave differently: some are weakly stimulatory, others inhibitory. Nickell *et al.* also assayed the activity of other purines. Hypoxanthine, xanthine and uric acid are slightly stimulatory at low concentrations.

Certain of the results obtained by Nickell on tumor tissue contradict those of Miller & Skoog (190) on normal tissues. In Nickell's experiments, xanthine and hypoxanthine are stimulatory, adenine inhibitory, and guanine inactive. Miller and Skoog, on the other hand, found that adenine and guanine counteract the bud inhibition produced by indoleacetic acid, but that xanthine and hypoxanthine are inactive. This comparison is of only limited scope since it concerns two processes which are not necessarily related: tissue proliferation and bud organization. The above-mentioned experiments are preliminary but show the interest of studies concerning the relation between plant tissue cultures and nucleic acid derivatives.

Other nitrogenous substances.—Other organic nitrogenous substances have been tested. Loewenberg & Skoog (183) used choline for the cultivation of normal pine tissues, but without saying what the effect was. Bouriquet obtained original results with mercaptoethylamine and mercaptothiazoline (23). These have the curious property of selectively stimulating the growth of crown gall tissue. Until now no definite compound was known possessing such activity if exception is made of the complex amino acid and vitamin mixture proposed by Paris & Duhamet (234). Gioelli has used dihydrofolliculine on carrot tissue; low levels are slightly stimulatory, but starting at a concentration of 1 to 40,000 it causes growth inhibition (108).

Antibiotics.—It may seem curious that antibiotics should be considered with the stimulating substances, since their practical applications are based on their inhibitory properties. The latter, however, concern mainly the bacteria, while animals and plants may react oppositely, i.e., may be favored in their development by weak concentrations. Preliminary tests performed by de Ropp in 1946 showed that various forms of penicillin stimulate normal sunflower tissues (252). But he used unpurified products with large amounts of indoleacetic acid, so that his results were doubtful. Later he used streptomycin on normal and tumor tissue (256) and obtained inhibition. In 1952, Nickell (212) using the pure products, showed that at low concentrations (5×10^{-6} to 10^{-5} gm. per ml.) penicillin, terramycin, streptomycin, thiolutin, and bacitracin considerably stimulated the proliferation of sorrel virus tumor tissue and could even replace vitamin B_{11}, which was considered essential for them. Nétien, Carraz & Sotty (205) also found that streptomycin stimulates the growth of carrot tissue. Camus & Lance (38, 41) studied the action of penicillin on normal Jerusalem artichoke tissues (which are incapable of proliferation without a division-stimulating substance) and on *Scorzonera* crown gall tissue (which proliferate spontaneously but are almost insensitive to auxins). Penicillin G used alone enhanced tissue proliferation in Jerusalem artichoke but had no effect on *Scorzonera.* At first sight, this effect seems related to that of auxin, However, the new tissue obtained in Jerusalem artichoke with penicillin is generally homogeneous instead of highly differentiated as with auxins.

Further, penicillin, unlike auxin, does not influence organogenesis. Its properties are not, however, exceptional as they resemble those of orange juice (Kovoor, see later).

Carcinogenic hydrocarbons.—Buvat (37) first tried carcinogenic hydrocarbons in plant tissue culture in 1942. He discovered that solutions of methylcholanthrene and benzopyrene in oil caused more exuberant calluses on carrot fragments than those obtained with indoleacetic acid. Some years later, Levine (174, 175, 176) made analogous studies on carrot, sunflower, and tobacco strains. He added benzopyrene, dibenzanthrene, and methyl cholanthrene to his media, and observed no generalized stimulation, but instead specific effects such as the development of buds (carrot and tobacco) and roots or tissue dissociation.

Coconut milk.—The use of coconut milk for *in vitro* cultures was proposed by van Overbeek, Conklin & Blakeslee (232) in 1942. These authors set out to obtain the development of certain *Datura* hybrid embryos which normally aborted. They supposed that abortion occurred because of albumen incompatibility due to its different genetic constitution and that the embryo might develop normally if separated from the ovule. Many attempts of this kind had failed in the past since the nutrient media did not allow good embryo growth. Van Overbeek, Conklin and Blakeslee thought that the liquid albumen which nourished the coconut embryo might be a good medium. Their attempt was completely successful.

In 1948, Caplin & Steward (44) cultivated carrot phloem fragments in media containing coconut milk. Growth was much better than that obtained with auxins. Autoclaving had almost no deleterious effect. They also found that the stimulatory activity is independent of seed age; even seedling extracts are active (275, 277). The spectacular nature of Caplin & Steward's results was due to: (*a*) the fact that they used a carrot variety almost insensitive to auxins; and (*b*) the use of small fragments whose proliferation initially demands more active substances than normal size explants [see Duhamet (64)]. Wiggans (301), working on the Chantenay variety whose tissues are very sensitive to auxins, has found that even here coconut milk is more effective.

Duhamet found that coconut milk strongly stimulates the proliferation of normal tissue from Virginia creeper, Jerusalem artichoke, and *Scorzonera* (56, 58). The newly-produced tissue differentiates less than that obtained in the presence of auxins (65). He also found that coconut milk enhances the growth of crown gall tissues (vine, tobacco, Jerusalem artichoke, *Scorzonera*), and of habituated tissues which are almost insensitive to auxins (57, 59, 60). The same is true for the virus tumor tissue of sorrel [Nickell (210)]. Copra extract causes cell multiplication in tobacco pith while auxins induce only cell enlargement [Jablonski & Skoog (139)].

Thus, the stimulatory properties of coconut milk are not due to their auxin content, but to a new substance which Steward and Caplin have called the coconut-milk factor (CMF). This factor, just as auxin, possesses the property of stimulating cambial cell multiplication but it also causes the proliferation of ordinary parenchymatous cells. Thus, Morel has used it to culture tissues of several monocots: *Amorphophallus*, *Sauromatum* (194), *Gladiolus*, *Iris* (195), etc. Nickell (personal communication) used it in the cultivation of *Agave* tissue. It is also employed for Gymnosperms [*Ginkgo*, Tuleke (285)] and dicots. Morel utilized it in his cultures of normal tissues of *Acontium fischeri* (195), *Ceropegia woodii*, tomato, tobacco, etc. (personal communication). Henderson, Durrel & Bonner used it for normal sunflower tissue (131), and Archibald for those of cacao (1). Bitancourt and his collaborators (personal communication) cultivate tissues of *Salix babylonica*, *Ruta graveolens*, etc., in media containing coconut milk. De Ropp *et al.* (259) and Caplin & Steward (personal communication) having failed to cultivate carrot tissue with auxin, used coconut milk.

The most effective concentrations vary from 5 to 20 per cent; those of 1 to 2 per cent are weakly active, while those above 20 per cent are toxic [Duhamet (58)]. In certain media both coconut milk and auxin are used. Thus, Levine (176) obtained particularly exuberant growth of carrot tissue in a medium containing both indoleacetic acid and coconut milk. Nickell cultivated normal *V. sinensis* tissues in a medium with coconut milk and *p*-chlorophenoxyacetic acid and those of *Rumex* in the presence of a mixture of coconut milk and 2,4-dichlorophenoxyacetic acid (personal communica-

tion). Steward & Caplin also used the synergistic effects of 2,4-dichlorophenoxyacetic acid and coconut milk to cultivate potato tissue (274). Studies of Duhamet (61) on *Scorzonera* and Jerusalem artichoke tissue have likewise given evidence of synergy between coconut milk and 2,4-dichlorophenoxyacetic acid or indoleacetic acid. These synergistic effects, however, are not observed with carrot tissue.

Although coconut milk possesses remarkable stimulatory and formative properties, it is not a panacea. Gautheret (103) found that normal tissues of Virginia creeper, rape-colewort and endive do not grow indefinitely in its presence. Proliferation is fast up to the sixth to tenth passage, but then diminishes and the cultures finally die.

Attempts to isolate the stimulatory substances from coconut milk.—The isolation of growth factors from coconut milk is of great interest since they are very active substances and have properties differing from those of either auxins or vitamins.

This isolation was undertaken for the first time by Shantz & Steward (264, 278). They started with 2,500 liters of coconut milk. A preliminary precipitation with mercuric acetate yielded a precipitate containing all the active principles. They were then dissolved to form a heavy syrup and subjected to a series of fractionations using solvents or chromatography. The activity of the various fractions thus obtained was determined by their action on carrot phloem tissue. There was finally isolated certain definite substances of controlled purity. Four were designated as A, B, C, and F and are active if combined with casein hydrolyzate. Shantz and Caplin determined their physical properties such as melting point, ultraviolet and infrared spectra, etc., but have not as yet established their formulae. More recent tests have been disturbing because the response of the tissues varies from one carrot to another (278). Further progress will therefore depend on the improvement of the biological test. This work, however, has been very fruitful.

Another attempt, but on a smaller scale, has been made by Skoog *et al.* (188). They used copra which also stimulates cellular multiplication. They started with an aqueous extraction, followed by elimination and denaturation of proteins. The active material was precipitated with mercuric acetate. Skoog *et al.* then electrodialyzed the precipitate and chromatographed it on paper. They thus isolated several substances giving amino acid reactions, but did not reach any conclusive results. They obtained a 4000 fold concentration of the copra principle, but did not succeed in isolating it in the pure state. Certain fractions are synergistic with indoleacetic acid. Finally, de Ropp, *et al.*, (259) have made a preliminary attempt but without any important result.

Therefore, the nature of the stimulatory substances from coconut milk remains unknown. In view of this, it was thought worth-while to prepare mixtures of known substances capable of showing analogous activity. The

first attempt of this kind was, as we have already seen, that of Paris & Duhamet (234). This attempt, though not entirely satisfactory, has nevertheless inaugurated an avenue of research which may prove fruitful.

Extracts from caryopses of Gramineae.—Following the remarkable success obtained with coconut milk, extracts from other monocots, such as the Gramineae, were investigated. In 1951, Nétien, Beauchesne & Mentzer (204) found that the liquid from young corn endosperm (corn milk) stimulates carrot tissue growth much more than does indoleacetic acid. This result was confirmed by Steward & Caplin (276) who demonstrated that the extract from ripe caryposes is inactive. Nétien and Beauchesne attempted the extraction of the factor contained in corn milk. A trial fractionation using sawdust revealed the existence of at least two active substances (202), but these were not isolated. In another attempt they used synthetic resins as adsorbants (203). All the active fractions thus obtained were less effective than corn milk and it appeared as if the properties of the corn milk were due to the combined action of many substances. Nétien and Beauchesne considered some of these substances to be amino acids but this has not been demonstrated.

Steward and Caplin discovered that the extracts from young caryopses of other grasses, such as barley, wheat, oats, and rye are also capable of stimulating proliferation of tissue cultures (276). With these, too, activity disappears at maturity. Their results with barley were confirmed by Skoog *et al.* (188). The extract from germinated barley (malt extract) is capable of stimulating tissue proliferation, especially that of tobacco pith parenchyma [Jablonski & Skoog (139)] and carrot tissue [Wiggans (301)].

Other extracts.—Certain extracts from seeds, fruits, or stems possess stimulatory properties. In 1951, Nitsch noticed that tomato juice, just as coconut milk, stimulates crown gall tissue proliferation (221). The following year, de Capite (43) obtained from tomato juice a marked effect on normal carrot tissue. Démétriades & Nitsch (55) published similar observations on the action of tomato juice on normal Virginia creeper tissue; but since their medium included indoleacetic acid they could not distinguish the respective role of the two components. Later research led to contradictory results. Thus, Démétriades & Nitsch (55) claimed that tomato juice inhibits the growth of certain tissues, particularly those of *Scorzonera* crown gall, while Kovoor (156) considers that it stimulates the proliferation. Straus and La Rue have taken advantage of the stimulatory and formative properties of tomato juice to cultivate successfully corn endosperm tissues (281).

Among other extracts from Phanerogams capable of stimulating the growth of tissue cultures can be mentioned those of young walnuts (*Juglans regia*), young *Aesculus* seeds, and the female gametophytes of *Ginkgo biloba* [Steward & Caplin (276)]. The ovary and young fruit of the banana tree also contain stimulating substances [Steward & Simmonds (279)].

Kovoor (153 to 156) undertook an extensive investigation on the juices extracted from the seeds of *Elais*, *Borassus*, *Allanblakia*, *Passiflora*, the

fruits of the orange tree, vine, *Artocarpus*, *Annona*, and the calices of *Spathodea*. These extracts show varied properties. Some stimulate the growth of both normal and tumor tissues. Others activate only tumor tissue. One of them, the intracalicinal liquid of *Spathodea* possesses the exceptional property of stimulating the growth of normal tissues in the same way as indoleacetic acid and of being inactive in the *Avena* test. Kovoor also found the histogenetic phenomena induced by these extracts to be very different. The most remarkable is that of orange juice which stimulates cell division in Jerusalem artichoke without determining differentiation. Six months after Kovoor's work on *Allanblakia* extract (154), Nitsch published the results of similar studies (222). His conclusions were the same although he could not determine the exact nature of the action of the extract as he always used it in conjunction with auxin.

Tissue cultures have also been treated with stem and leaf extracts. Wiggans (301), for example, found that the juice of ground carrot leaves stimulates the proliferation of this species, even if the indoleacetic and other organic acids it contains are previously eliminated. On the contrary, root extracts are toxic. Curious results were obtained by Jablonski & Skoog on tobacco pith parenchyma (139). Isolated fragments of this tissue are unable to proliferate spontaneously. In an auxin medium cells enlarge without dividing, but if one places stem fragments containing xylem together with pith fragments in an auxin medium, then the latter proliferate as if in the presence of coconut milk.

Extracts from tumor tissues.—The fact that tumor tissues, particularly those of crown gall, proliferate very actively suggested the presence of division-stimulating substances in them. The first attempt to discover whether tumor extracts would affect cultures was that of Hildebrandt, Riker & Duggar (135) in 1946. Juices obtained by expressing crown gall from tomato, *Tagetes erecta* or *Chrysanthemum frutescens* strongly stimulate sunflower crown gall tissues, but are only slightly active on the genetic tumors of tobacco. This stimulation cannot be due to an auxin since auxins do not affect the growth of tumor tissues, but appears to be a factor with the same physiological properties as coconut milk. This conclusion drawn from old experiments of Hildebrandt, Riker and Duggar is confirmed by the recent work of Braun & Naf (33). They compared the effects of auxin and of crown gall extracts on tobacco pith fragments. Auxins caused only cell enlargement, in accordance with the results of Jablonski & Skoog (139), while crown gall extract was almost inactive. But by combining these two substances, Braun and Naf observed an exuberant proliferation of the explants. This result is very interesting, since it shows that tumor proliferation necessitates not only the intervention of an auxin, a fact which was already well-known [Kulescha & Gautheret (164); Henderson & Bonner (130)], but also a substance of a different type. One cannot be sure that this factor is specific for tumor tissues since Jablonski & Skoog (139) obtained proliferation of tobacco pith fragments with extracts of tobacco vascular tissues taken from normal plants or, again,

with copra extract. Moreover, the stimulatory properties of crown gall extracts are not general. Hirth & Ryback (137) found that extracts of *Pelargonium* are always toxic.

Extracts from cryptogams.—Yeast extract was used for the first time in 1922 by Robbins for the culture of isolated roots (249). White utilized it with great success (288) and with it obtained the indefinite cultivation of tobacco tumor tissue (290). Hildebrandt, Riker & Duggar (135) evaluated quantitatively its stimulatory properties. It is strongly active on the growth of sunflower crown gall but only moderately so on tobacco tumor tissue.

Even though the composition of yeast extract is poorly defined and other substances are known to be active, it is still currently used for the cultivation of various tissues. Straus & La Rue (281) claim that it is essential for the development of corn endosperm tissue cultures. Its activity is reduced 40 per cent by autoclaving.

Sussex & Steeves (283) also used yeast extract for the cultivation of the tissues of *Pteridium aquilinum*, although its essentiality is doubtful. Nickell (personal communication) uses it currently in his cultures of melilot and of *V. sinensis*, but in conjunction with auxins and even with coconut milk. A mixture of auxin, yeast extract, and coconut milk was resorted to by Tulecke (285) for the isolation of a stock of *Ginkgo* tissue. Jagendorf & Bonner (147) consider this extract to stimulate the growth of cabbage tissue.

The question of the nature of the active substances contained in yeast extract has been examined. Root culture work [Bonner (21, 22); Robbins & Bartley (250); White (289)] emphasizes vitamin B_1. But this vitamin is rarely active in tissue cultures. According to Straus & La Rue (281) an essential role must be assigned to the amino acids. The extracts of other fungi are equally active. Thus, Jacquiot showed that extracts from *Phellinus robustus*, which commonly develops on oak, are capable of stimulating the proliferation of Jerusalem artichoke tissues (145). This activity is related to that of auxins, but it is doubtful whether only indoleacetic acid is involved. The extracts from the crown gall bacteria, *Agrobacterium tumefaciens*, have been studied by Riker & Duggar (135), who could obtain no stimulation.

Other experiments by Gioelli (107) have shown that the filtrate from *A. tumefaciens* cultures stimulates the proliferation of the cambial tissue from *Sterculia platanifolia*. Maybe this phenomenon is caused merely by the presence of auxin.

Extracts of animal origin. Protein derivatives.—We shall examine successively the activity of protein derivatives, of insect secretions, and finally of chick embryo extracts. The encouraging results obtained with amino acids suggested the use of certain degradation products of proteins, such as peptones and casein hydrolyzate, for the cultivation of plant tissues.

Peptones were used more than fifty years ago by Haberlandt (119) during his first investigations on cell culture. In 1922, Kotte (152) used them to cultivate root meristems. All these old experiments were unsuccessful. The

only encouraging result has been recently obtained by Kandler (149). He was able to cultivate normal sunflower tissue in a medium containing Witte peptone. Henderson, Durrel & Bonner, however, cultivated tissue from the same species in a medium without peptone (131). However, they used other complex substances rich in amino acids such as coconut milk and casein hydrolyzate. This last product is particularly interesting. Henderson *et al.* (129, 131) have shown that the proliferation obtained with it is only surpassed by that induced by coconut milk. On the contrary, the hyrolyzate is toxic for those tissues which have undergone habituation.

Casein hydrolyzate was also used by Morel to cultivate *Ginkgo* tissue, as well as that of certain Selaginelles and certain ferns (personal communication). Straus & La Rue (281) believe it can replace yeast extract for corn endosperm tissue culture.

The active substances isolated from coconut milk by Shantz and Steward only manifest their stimulatory and formative properties in the presence of casein hydrolyzate. Thus, the hydrolyzate is very active in promoting tissue proliferation. This is not surprising as it is known that amino acid mixtures stimulate tissue culture growth [Paris & Duhamet (234)]. But the activity of these mixtures has not yet been analyzed and it is not known what combination of amino acids is particularly effective.

Insect secretions.—The tissues abutting on the borings of insects in trees sometimes show signs of proliferation. This is true of the lesions caused on oak by *Agrilus biguttatus* [Jacquiot (142)]. The content of the borings yields an alcohol-soluble factor inducing Jerusalem artichoke tissue proliferation. Chromatographic fractionation undertaken by Gouvernel-Guillemain (118) has shown that the stimulatory factor is really a mixture of two substances. According to Jacquiot & Gouvernel-Guillemain (146), one of them is dialyzable and alcohol- and ether-soluble, while the other is nondialyzable and is alcohol- or benzene-soluble. Their nature has not been determined. It is quite probable that numerous other coecidia, producing insect larvae, produce division-inducing substances.

Chick embryo extracts.—Nystérakis (230, 231) had the original idea of cultivating plant tissues in media containing chick embryo extract. This extract slightly stimulates isolated carrot cuttings if no auxin is present. With indoleacetic acid, it attenuates the stimulatory and formative properties of the auxin. Nystérakis further found that autoclaving conferred upon it inhibitory properties. These results are difficult to interpret in view of the extremely complex nature of the extract from chick embryos. The observed stimulation may very well not be due to any division-stimulating substance. It can be simply a nutritional effect, since the extract is rich in many compounds of biochemical importance. Shantz and Steward[4] obtained negative

[4] Remarks made at the First Symposium on plant tissue culture [See Nystérakis (231)].

results with chick embryo, liver, tumor tissue, and aminotic liquid extracts. It is thus improbable that this type of work will be successful.

Variations in Growth Factor Requirements

We mentioned (see above) that the requirements for growth factors (auxins, vitamins, coconut milk, etc.) vary according to the species and even according to the nature of the tissues from one and the same species. Further, the requirements also change with time and with the size of the explant. One can distinguish reversible and permanent modifications.

Reversible modifications.—The best example of reversible modifications was observed by Gautheret (102, 106) and by Kulescha & Gautheret (162) on Jerusalem artichoke tissues. Tissues taken from young rhizomes in autumn or from germinating tubers in spring are capable of proliferating spontaneously, while explants excised in winter need auxin for growth. This difference is temporary, since after two to three months in culture, the tissues require auxin no matter what their origin.

Permanent modifications.—Tissues can manifest either an increase or a decrease in their nutritive requirements. Gautheret observed increases in nutritive requirements of Jerusalem artichoke and *Scorzonera* normal tissue cultures (103), and also of the carrot strain isolated by Levine (106, 176). These tissues can be cultivated in a medium containing auxin as the sole growth factor. If one transfers into media containing coconut milk, proliferation is accelerated. If this substance is then withheld, the growth of the tissue ceases almost completely. Having been subjected to coconut milk, the tissues can no longer maintain growth without it.

Normal *Scorzonera* tissues cannot be grown without a growth factor, but they develop indefinitely in the presence of weak auxin concentrations (88). But Gautheret (106) has observed that some tissues, having undergone numerous transfers, acquire the faculty of growing without auxin and furthermore become completely insensitive to this substance. An analogous phenomenon occurs with normal carrot tissue. Since these are originally auxin-independent all that occurs is a loss in sensitivity to these substances (81).

Gautheret has given the name of "accoutumance à l'auxine" to this curious phenomenon (American authors translate it as "habituation"). Later, (105, 106) he proposed the expression "anergie à l'auxine" (from the Greek *αν* meaning privative, and *εργ* meaning to act) which has the advantage of stressing the fact that the stimulating substance ceases to be active.

Following these original observations, the phenomenon has been noted many times. Morel (193) observed it in tissue cultures of vine, Virginia creeper, tobacco, and mallow. Nickell found it in melilot; Kandler (149) and Henderson (129) in sunflower; Hildebrandt and Skoog on tobacco (personal communications); Galston on *Scorzonera* (personal communication); and Czosnowski (53) in carrot. Apart from the particular behavior towards auxin the "anergized" tissues possess a group of properties which allow them to be

distinguished from the corresponding normal tissues. They are transparent and friable, incapable of root formation [Gautheret (81, 106)], and their concentration of free auxin is high [Kulescha (159)]. Their capacity for differentiation is usually weak [Morel (193); Henderson (129); Czosnowski (52)].

They show more or less pronounced tumor-forming properties, as evidenced by grafting. In *Scorzonera* [Camus & Gautheret (39, 40)], sunflower [Henderson (129)] and particularly vine [Braun & Morel (32)], the tumors obtained by grafting habituated tissues are less exuberant than those given by grafts of crown gall tissue. On the contrary, in tobacco the habituated tissues are more malignant than the crown gall tissues [Limasset & Gautheret (177)]. Systematic studies carried out by Czosnowski (52) on vine tissues revealed that anergized tissues differ from normal ones by a host of biochemical characters, for example, their protein-N content, soluble sugars, enzymes (proteases, amylase, catalase, etc.). These differences are more accentuated than in crown gall tissues. It appears that the metabolism of anergized tissues is even more aberrant than that of crown gall tissues. This conclusion is only valid, of course, for vine tissues. In addition, as already discussed, the reactions of anergized tissues towards certain growth factors is very different from corresponding normal tissues.

The investigations of Gautheret (106) have shown that there are degrees of anergy. It manifests itself only in growing tissues. It can be obtained without auxins in the medium, but substances of this type greatly favor it (10^{-6} to 10^{-5} gm. per ml. indoleacetic or naphthaleneacetic acids are the most effective). Many short duration treatments are better than a single long one. Gautheret considers it to be the result of a profound modification of the chemistry of the cell, for example, deviations in protein metabolism (106). This modification is neither mutation nor selection, but rather a kind of enzymatic adaptation (106).

Another type of reduction of nutritional requirements is known involving vitamins. To isolate a stock of willow tissue, Gautheret (96) used vitamin B_1, biotin, and pantothenic acid, the latter of which thereafter ceased to be needed. It appears as if this were a phenomenon of anergy to pantothenic acid. Facts of this type have been also observed by Morel and Jacquiot (personal communications). These various modifications of nutritional requirements cannot be overlooked when using tissue cultures in physiological investigations.

Variability of nutritional requirements as a function of explant size.—It has been known for a long time that the growth of certain microorganisms depends on the size of the innoculum. This peculiarity was the theme of a historical controversy between Pasteur and Liebig. The former, using large innocula, obtained different results with yeast than Liebig who used extremely small ones. Wildiers (302) cleared up this contradiction by showing that yeast requires growth factors called bios which are poorly represented in small amounts of yeast.

Gautheret performed analogous observations (83) on carrot tissue cultures. Explants weighing at least 30 mg. developed regularly, while the start of proliferation was irregular if the amount of tissue was smaller. Duhamet studied the details of this problem (62, 63, 64). He established that the threshold size below which tissues are incapable of growing depends on the amount of growth factors supplied externally. He used a carrot strain capable of proliferating spontaneously if the size was at least of 10 mg. With 10^{-10}, 10^{-9}, or 10^{-8} gm. per ml. indoleacetic acid the weight limit could be brought down to 5, 2.5, and 1 mg., respectively. With 10^{-6}, the size could be lowered even more to 0.5 mg. Results are similar with 2,4-dichlorophenoxyacetic acid. Duhamet observed an identical phenomenon in anergized tissues of *Scorzonera* (63) which are almost insensitive to auxin with large fragments$_1$ (100 to 200 mg.); the weight must be at least 16 mg. to get the colonies started if the medium is deprived of auxin; with 10^{-8} gm. per ml. indoleacetic acid, the threshold falls to 0.5 mg. Crown gall tissues of the same species yielded uncertain results (64).

Guioneau-Besset [see Duhamet (64)] demonstrated that small fragments exhaust their auxin content so quickly that cultures cannot be started unless an external auxin supply is furnished. Duhamet obtained similar results with coconut milk. This substance not only helps start cultures but stimulates proliferation as well.

Yeast extract yields similar though not as clear-cut results. Vitamin B_1, meso-inositol, and biotin, when supplied simultaneously, exert no influence on growth; in conjunction with indoleacetic acid they do not make possible a decrease in the size of the explants but they reinforce the proliferation induced by the auxin. The same vitamin mixture increases the stimulatory and formative activity of coconut milk [Duhamet (64)]. It is thus seen that nutritional requirements depend on explant size, and that while small fragments may require auxins or coconut milk, larger ones may develop spontaneously.

The cultivation of small explants has resulted in attempts to cultivate individual cells. The early investigators of tissue culture tried in vain to solve this problem. Schmucker (260) in 1925, and later Northcraft (229) in 1951 claimed success but their results have not been confirmed. Recently, Muir, Hildebrandt & Riker (199) made fruitful trials using a very original technique. This consists of placing the isolated cells on filter paper tightly appressed to a tissue colony. The cells have at their disposal the liquid exuding from the tissues which enables the cells to proliferate. This work is very interesting but there remains the problem of the nutritive requirements of the isolated cells. For example, what is the nature of the liquid exudate of the colonies?

Growth Inhibitors

The tissue culture method has been used to analyze the properties of certain inhibitory substances, especially the antagonistic effects which they sometimes show towards stimulating substances. We shall consider succes-

sively the inhibitors of definite composition and those of natural origin whose constitution is ill-defined. We shall examine first the properties of hydrazides; then those of other compounds.

Hydrazides.—Maleic hydrazide has been the object of numerous experiments. The first ones on tissue cultures were those of Bertossi (8, 11, 12, 13), and Gautheret (99, 100). At suitable concentrations, maleic hydrazide inhibits the development of normal tissues of carrot, endive, and Jerusalem artichoke. Nickell (215) found that it also inhibits the growth of both normal and sorrel tumor tissue. Kulescha made analogous observations on crown gall tissue of *Scorzonera* (160). Bertossi obtained growth inhibition in anergized tissue of Virginia creeper. The experiments of Gautheret with Jerusalem artichoke (99) as well as those of Bertossi & Capozzi (11, 12, 13) established that maleic hydrazide behaves as an antagonist of indoleacetic acid. It acts not only against the stimulatory and formative activity of the auxin but also attenuates the toxicity of supraoptimal levels of indoleacetic acid. Furthermore, at weak concentrations maleic hydrazide acts synergistically with indoleacetic acid. The same antagonism is shown against 2,4-dichlorophenoxyacetic acid or naphthoxyacetic acid (Bouriquet, personal communication). The examination of histological sections reveals that maleic hydrazide alters histogenesis [Gautheret (102)] and disturbs particularly the processes of lignification [Jacquiot (144)].

The remarkable properties of maleic hydrazide have stimulated research on other hydrazides. Goris & Phouphas (117) found that isonicotinic hydrazide is also inhibitory. Bertossi (11) working with Jerusalem artichoke and carrot tissues observed a slight antagonism between isonicotinic hydrazide and indoleacetic acid. On the contrary, α-naphthaleneacetic hydrazide is not inhibitory and behaves almost like naphthaleneacetic acid itself [Bouriquet, Goris & Muller (27)].

Antimitotic substances.—Some investigators have tried to disturb the development of plant tissues by means of antimitotic agents which, as is well known, paralyze the course of mitosis. The first work was done by Martin with colchicine (186, 187). Using it in conjunction with indoleacetic acid, he found that at low concentrations (10^{-6} gm. per ml.) the stimulation of the auxin was enhanced, while at higher levels it arrested growth;[5] mitoses aborted and large, lobed nuclei were formed in enlarged cells. When the colchicine was removed, the large nuclei fragmented and dividing walls appeared giving rise to small cells in the midst of hypertrofied elements. The stimulating and inhibitory action of colchicine have been further studied by Nickell in his researches on the physiology of tumors of viral origin (209). Capozzi (46) showed that two other antimitotic substances, isopropyl-phenylcarbamate and γ-hexachlorohexane, have a depressing effect on the growth of carrot tissues.

[5] The stimulating action of low concentrations of colchicine is not specific; most toxic substances behave in the same way at weak doses.

Anticancerous substances.—De Ropp (258) tested the response of normal and tumor tissues to some of the substances considered to be inhibitors of animal cancer. Some of these substances, especially numerous pterins, inhibit normal and tumor tissues, but the former more effectively. These inhibitors are thus not specific for tumor tissues. Certain purine analogues, to which anticancerous properties are attributed, also inhibit the growth of virus tumor tissue as, for example, guanazol, various guanine derivatives, 2,6-diaminopurine, 6-mercaptopurine, etc. [Nickell (217)].

Miller made a detailed study of one of these natural purine analogues, 2,6-diaminopurine (189). He found that it impedes the proliferation of normal and anergized tissues of tobacco and also the enlargement of pith parenchyma cells. Its inhibitory properties are counteracted by adenine and less so by guanine and hypoxanthine.

Aniline dyes.—It is known that certain aniline dyes hinder virus development. Nickell (211) found some of them (methylene blue, crystal violet, malachite green) to inhibit strongly virus tumor growth. Neutral red is only weakly inhibitory. Others, such as trypan blue, pyronin, azur A and methyl green stimulate proliferation at low levels but are inhibitory at higher concentrations. We are not dealing here, however, with a specific effect, as these dyes do not destroy the virus.

Antivitamins.—The only antivitamin that has been used is *p*-aminophenylsulfamide. De Capite (42) cultivated normal Jerusalem artichoke and *Scorzonera* crown gall tissues in a medium containing a mixture of the antivitamin and the corresponding vitamin, i.e., *p*-aminobenzoic acid. He found that at low concentrations the sulfamide reinforces the stimulatory action of *p*-aminobenzoic acid, while at higher concentrations it behaves as its antagonist. This is in accordance with classic concepts.

Other substances.—Other inhibitors of the most varied type have been used. One of them, 2,3,5-triiodobenzoic acid had been used many times on whole plants and its influence on flowering is well known. Nickell showed in 1950 (209) that it is a powerful inhibitor of the growth of sorrel virus tumor tissues. Niedergang (220) has shown that at lower concentrations it does not inhibit growth, but instead suppresses the polarized circulation of auxin in such a way that newly-formed calluses and organs (for example, buds) appear disseminated over the explant.

Another inhibitor, naphthylphthalic acid, also shows curious properties. At high levels it blocks completely the growth of colonies, but at certain concentrations it changes the normal patterns of histogenesis and causes the formation of phloem and xylem in an inverse position to that found in normal stems [Gautheret (102)].

Jacquiot (140) studied the inhibition by tannins. Having found that certain tissues rich in tannin (oak, chestnut) are incapable of proliferating *in vitro*, he thought that this might be due to the tannins. He tried cultivating them in media containing caffein, which precipitates tannins. He succeeded completely, for the tissues then grew satisfactorily.

In the discussion of carbohydrate nutrition and growth factors it was noted that certain carbohydrates, alcohols, organic acids, etc., have inhibitory properties. The same is true of some degradation products of nucleic acids, such as adenylic acid, adenosine, etc. [Nickell, Greenfield & Burkholder (219)].

Ill-defined substances.—The investigations carried out on plant extracts revealed the existence of substances capable of elliciting stimulative and formative effects. Very often, however, these extracts show inhibitory properties. Steward & Caplin (275) found, for example, that extracts from castor oil plant seeds, fruits of *Fagopyrum*, potato tubers, onion bulbs and maple tree buds inhibit the proliferation of carrot phloem, even in the presence of coconut milk. According to them, the inhibitory substances would accumulate in those organs which have finished their development, while young organs would contain instead stimulating substances.

There is no evidence indicating that these are really specific inhibitions. These extracts may be toxic to carrot cells because they belong to widely different species, but they might stimulate the tissues from which they have been extracted. Autoinhibition of a given tissue by its own extract is, however, known. Wiggans (301) showed that carrot root juice inhibits the proliferation of carrot tissues, while leaf extracts are stimulatory. Hirth & Ryback found that *Pelargonium* extracts are toxic for the tissues of this species (137) and according to them the presence of tannins does not wholly explain this toxicity. It should be remembered, however, that in certain cases tannins are in themselves inhibitory, and that their inhibition can be overcome by caffein [Jacquiot (140)]. Melanin, which forms as a consequence of wounds is also inhibitory of the proliferation of certain tissues (Morel, personal communication).

Finally, certain stimulatory extracts contain inhibitors which can be made conspicuous by fractionation, for example, by chromatography. In the course of their fractionation of corn endosperm extract Nétien & Beauchesne (202) showed the existence of two stimulants and one inhibitor of Jerusalem artichoke tissue. Bitancourt *et al.*, in the same way, revealed the presence of growth inhibitors in tobacco and willow tissues (16, 17). These studies have only started the investigation of a vast domain and it is probable that division inhibitiors are as common as division stimulants.

Effect of Growth Regulators on the Metabolism of Plant Tissue Cultures

There are close relations between growth and metabolism. Factors capable of influencing one should modify the other. Some phases of metabolism have been considered to be affected particularly by growth regulators. The method of *in vitro* culture lends itself beautifully to this type of study and has been, therefore, taken advantage of in several circumstances.

Carbohydrate metabolism.—We shall examine the effect of stimulatory substances such as auxins and coconut milk and of an inhibitor, maleic hydrazide.

Stimulatory substances bring about in a general way the mobilization of carbohydrates, but their effects on permeability and respiration complicate the general picture. Cova (50) and Goris (111) were the first to establish that carrot tissue cultivated in a sugar-free medium gradually depletes its carbohydrates. Auxins considerably accelerate this depletion [Goris (109)] because, it appears, of their action on proliferation and respiration (see later). This is observed not only in carrot tissues but also in those of Jerusalem artichoke (112). This depletion is particularly striking if related to fresh weight since auxin favors the hydration of the tissues [Goris (110)].

If the medium contains sugar such as glucose, sucrose, or fructose, this is absorbed, thus reducing the internal carbohydrate consumption or even resulting in a more or less transitory accumulation within the tissues [Cova (50); Goris (115); Lioret (182)]. Here, one generally finds that auxins instead of acting in favor of sugar depletion causes their accumulation, which can be explained in part by a change in cellular permeability. This idea is not arbitrary since many workers have shown that auxins increase the permeability of cells to water [Reinders (245); Hackett & Thimann (121), etc.] or to mineral salts [Heller (126); Brunstetter *et al.* (34)]. The relative nature of this auxin-induced sugar accumulation has been investigated. Skoog & Robinson found for tobacco tissue that only reducing sugars accumulate (269). Arabinose, which was absent from the controls, is formed, probably as a result of the degradation of pectic compounds in connection with cell growth. Wiggans also obtained accumulation of reducing sugars (glucose and fructose) in carrot tissue (301). Working on the same material, Goris found, on the contrary, that indoleacetic acid causes an accumulation of all sugars, both reducing and sucrose (115). Some investigations of Lioret on *Scorzonera* normal tissue have yielded different results, since auxin, instead of favoring sugar accumulation accelerates metabolism. Crown gall tissue, the growth of which is not affected by auxin is equally unaffected in its carbohydrate metabolism [Lioret (182)].

The action on carbohydrate transformations of other cell division stimulants has also been studied. In 1950, Goris found that coconut milk causes carbohydrate depletion, especially of reducing sugars, which can even disappear completely (113). In 1954, Wiggans confirmed Goris's observations but could not detect a complete disappearance of reducing sugars (301). He, furthermore, showed that adenine has the same effect. It can be seen, therefore, that stimulatory substances have different effects on carbohydrate transformations.

Investigations on whole plants by MacIlrath (185), Wittwer & Hansen (306), and Naylor (200) showed that plants inhibited by maleic hydrazide become richer in carbohydrates, particularly sucrose. The technique of *in vitro* cultures has been applied to the study of this phenomenon. Pouphas & Goris (238) discovered that in Jerusalem artichoke tissue maleic hydrazide causes the transformation of inulin into sucrose. This undoubtedly results from a modification in the enzymatic systems within the tissues. For carrot,

Pouphas also found an increase in sucrose, at least at the beginning of cultivation (237). Maleic hydrazide appears also to interfere with the isomerization of glucose to fructose. Goris & Bouriquet (116) further noted a curious phenomenon. At low concentrations as well as at high ones (10^{-8} to 10^{-7} and 10^{-4} gm. per ml.) accumulation of sucrose occurs, but intermediate levels (10^{-6}) have no effect. However, close analysis of the results show that the sucrose accumulation noted at high concentrations is illusory since colonies do not develop. Therefore, only the weak concentrations are active. They also showed that auxin does not modify this effect. Similar researches were undertaken by Richez & Gautheret (246) on sugar beet cultures. Here, maleic hydrazide induces only a slowing down of the disappearance of sucrose.

The sucrose-forming capacity cannot be attributed to the hydrazide alone, since isonicotinic hydrazide has no effect on the carbohydrate composition of the tissues [Goris & Phouphas (117)]. *Alpha*-naphthaleneacetic acid hydrazide behaves like the acid [Bouriquet, Goris & Muller (27)].

Growth regulators and respiration.—Much work has been done on the influence of growth factors on respiration either in whole plants, or in organ fragments incapable of developing indefinitely (*Avena* coleoptile, pea epicotyl, etc.). The studies on the respiratory changes in tissue cultures are fragmentary. The first was done by Plantefol in 1938–39 (239, 240, 241). He found that excision causes intense oxidative activity. He compared the respiration of the newly-formed and the pre-existent tissues and ascertained the influence of glucose on the respiratory exchange. Later, White (294) compared the respiration of normal and crown gall tissues, and concluded that the tumor transformation is accompanied by a decrease in cellular oxidations, in accordance with the change which occurs in animal tumors.

Lachaux was the first to study the influence of growth regulators on tissue culture respiration (169 to 172). He established that indoleacetic acid increases respiration slightly in normal carrot and Jerusalem artichoke tissues, with a maximum effect at 10^{-6} gm. per ml. His data did not permit distinguishing between a direct action or a reflection of tissue proliferation which, as we know, is stimulated by auxin.

Nickell confirmed these results (209). He worked with tumor tissues of viral origin, which are only slightly affected by auxin, so that the stimulation in gaseous exchange observed could be attributed to a certain degree to auxin. With indoleacetic acid, the concentrations giving optimum growth and highest respiration were coincident. With 2,4-dichlorophenoxyacetic acid and naphthoxyacetic acid, the maximum for respiration was attained at levels much higher than those giving optimum growth. He noted, too, that the effect of auxins on respiration could not be specific, since he obtained similar effects with inhibitors such as triiodobenzoic acid and colchicine.

Mitchell, Burris & Riker (191) studied the effect of auxins and certain other substances on the respiration of different organ fragments. They observed mostly inhibitory effects, but this appears to be due to their constant use of high concentrations. A bit later, Eberts, Burris & Riker (68) per-

formed similar experiments on normal and crown gall tomato tissue. But this time, too, they used massive concentrations of auxin, and found only a non-specific inhibition which could not be counteracted by any of the Krebs cycle acids.

Lioret, also, compared the respiration of normal and tumor tissues (178 to 182). He used *Scorzonera* and weak auxin concentrations. Naphthaleneacetic acid gave an increase of gaseous exchange in normal but not in crown gall tissues. Coconut milk, on the other hand, induced higher respiratory rates in both types of tissues. By measuring growth and protein-N he determined that the metabolic modifications induced by the two substances have different origins. Coconut milk induces intense protein formation, the increase in respiration being thus probably due to an increase in living matter. Auxin, on the other hand, has practically no effect on protein synthesis, so that the higher gaseous exchanges are here a true expression of enhanced cytoplasmic catabolism. Lioret also discovered an interesting effect of coconut milk on *Scorzonera* crown gall tissue. This substance causes during the first week of cultivation an enhancement of respiration, then a decrease associated with intensified protein synthesis and growth. Studies of this type comparing different aspects of metabolism are evidently very instructive. The same is true of the investigations concerning the energy balance of cultures which have been inaugurated by Kandler on anergized vine tissues (150).

Finally, some investigators have studied the effect of auxin on the respiration of organ fragments under conditions in which proliferation was precluded. Newcomb worked on tobacco pith fragments where cells enlarge under the exclusive action of auxin (206). Hackett & Thimann (120) used Jerusalem artichoke discs and completed their experiments before proliferation was initiated, i.e., after six days. In both cases, indoleacetic acid stimulated respiration. Therefore, the effect of auxin appears to be direct and one cannot consider it as a simple consequence of the growth which it induces. However, a superposition of both processes is very possible.

Growth regulators and respiratory enzymes.—The results obtained with tissue cultures have encouraged investigators to go further in probing the details of cellular oxidation and the behavior of the enzymes involved.

In 1951, Newcomb (206) showed that the increased respiration of tobacco pith fragments caused by auxin was correlated with enhanced ascorbic acid oxidase activity. He also found that the enzyme was localized at the membrane or close to it and concluded that there must be a relationship between ascorbic acid oxidase, cell enlargement and respiration. The cells of his explants, however, did not divide, but only enlarged, so that his work does not directly bear on tissue culture.

Morel (196) recently began similar studies on other oxidative enzymes. He discovered that peroxidase activity in normal Jerusalem artichoke tissue is increased by indoleacetic acid and that it is in relation to proliferation. Camus & Lance (41) obtained similar activation with penicillin G. Maleic

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hydrazide, which inhibits colony growth, decreases peroxidase activity [Morel (196)]. At first sight one might think that peroxidase activity is a direct consequence of growth, but this cannot be generalized since, according to Morel, 2,4-dichlorophenoxyacetic acid which considerably enhances proliferation, has little effect on peroxidase activity.

He also studied polyphenoloxidase. Indoleacetic acid and 2,4-dichlorophenoxyacetic acid cause enhanced activity in Jerusalem artichoke tissue cultures. But maleic hydrazide, which inhibits growth, also activates the enzyme. Here, again, there is no relation between enzymatic activity and proliferation. In the case of dopa-oxidase studied by Camus & Lance (41), one finds a higher activity induced by penicillin, while indoleacetic acid has the reverse effect.

Finally, the effect of growth inhibitors on catalase activity has been studied. Galston (74) to whom we are indebted for the first work on this subject, found plant tumor tissue, just as that of animals, to be characterized by a low catalase concentration. He determined the catalase activity of normal, anergized, and crown gall tissues of several species. As a result of further experimentation he concluded that catalase activity depends inversely on the rate of proliferation, rather than on the nature of the tissue. Thus, indoleacetic acid and 2,4-dichlorophenoxyacetic acid, which stimulate the development of normal tissues of Jerusalem artichoke, lower catalase activity. Morel (196) confirmed these results but not the generalization, since maleic hydrazide, which strongly inhibits growth, causes a decrease in the enzyme activity, contrary to what would have been expected. Camus & Lance (41) also obtained contradictory results in their studies of catalase activity in penicillin G and indoleacetic acid-treated tissues. These preliminary results are contradictory and further work is urgent.

Growth regulators and other enzymes.—Studies on tissue culture have occasionally led workers to study the influence of growth regulators on various enzymes. Brakke & Nickell, (29, 30) for example, studied the amylase secreted by the virus tumor tissues of sorrel. They cultivated these tissues in media containing indoleacetic acid, 2,4-dichlorophenoxyacetic acid or β-naphthoxyacetic acid, and then after removal of the colonies they evaluated the amylase contained in the substratum. No difference relative to the controls could be observed.

Bryan & Newcomb (35, 207) studied the pectin-methylesterase produced by tobacco pith parenchyma. Under the influence of indoleacetic acid, the parenchyma cells grow, entailing a synthesis of pectic compounds from soluble uronides. This synthesis is stimulated by indoleacetic acid [Wilson & Skoog (305)] and this is linked with an increase in pectin-methylesterase, an enzyme participating in the metabolism of pectic compounds [Bryan & Newcomb (35)]. These results are still fragmentary, but they show how *in vitro* cultures can be of service in the study of the enzymatic mechanisms involved in cell growth.

Growth regulators and nucleic acids.—The phenomena of proliferation

taking place in tissue cultures evidently entail modifications in the nucleic acid metabolism, and it has been thought with reason that this process should be influenced by stimulatory substances, especially auxins.

Silberger & Skoog (265, 266) found that indoleacetic acid determines the formation of nucleic acids by isolated tobacco pith fragments. Both desoxyribonucleic and ribonucleic acids are involved. This fact may be surprising at first sight since auxin causes only the enlargement of cells, not their division. But it must be borne in mind that enlarging cells are sites of mitoses, which brings about an increase in nuclear material.

Growth regulators and viruses.—Finally, some investigators have studied the effect of growth regulators on the viruses associated with tissue cultures. Preliminary studies were done by Augier de Montgremier, Limasset, & Morel (2) on the maintenance of viruses in tobacco tissue cultures. Their results were verified by Kutsky and Rawlins. They found that naphthaleneacetic acid [Kutsky & Rawlins (168)] or indoleacetic acid [Kutsky (167)] produce a notable decrease in the virus content of tobacco tissue affected with mosaic. On the other hand, phenylacetic acid and other compounds such as phenylpropionic acid, ribonucleic acid, streptomycin, etc., are without effect [Kutsky (167)]. Segrétain, working on tobacco crown gall tissues affected by mosaic, found, on the contrary, an increase in the virus under the influence of naphthaleneacetic acid (262). Coconut milk stimulates even more the production of virus.

Segrétain and Hirth (265) also studied the action of various nitrogen compounds on mosaic virus in tobacco tissue culture. Aspartic acid decreased virus multiplication, while glutamic increased it. Proline, histidine, etc., were indifferent.

Liquid and Solid Media

The early investigators of tissue culture found that colonies developed poorly in liquid media and that it was necessary to place them on the surface of agar media. According to White (291), immersion causes asphyxia, while, according to Gautheret (98), the mere contact of the medium would impede proliferation, at least of willow tissues.

The necessity of using agar media is inconvenient in many nutritional studies, since agar contains impurities. In certain cases, therefore, liquid media are imperative. To obviate asphyxia, mechanical devices allowing intermittent immersion can be used [Caplin & Steward (45); de Ropp (253)]. Another way is to shake continuously the flasks containing the colonies, thus permitting a convenient influx of air [Morel, personal communication; Jagendorf & Bonner (147)]. Heller & Gautheret (128) simply used glass supports which maintain the tissues on the surface of the liquid. Later, Heller (124, 126) used filter paper supports which have many more advantages. White (298) compared growth in agar medium and in liquid medium with intermittent immersion. He found that proliferation is faster in liquid medium at the beginning, but the reverse is true by the end of one month.

This comparison is interesting from a practical standpoint, but no general conclusions could be drawn since, in one case, the tissues were intermittently in contact with air while in the other the contact was continuous. In Heller's experiments, on the contrary, the explants were always at the surface of the medium whether this was solid or not. In this case, solid media yielded consistently better results. Heller (125, 126) also established that other colloidal gels, such as silica-gel give better results than liquid media. Agar would thus act not only as a provider of nutrients, but its benefical action would at least in part be due to its colloidal nature.

Conclusions

The first studies on the nutrition of tissue cultures were of a rather pragmatic nature, since their essential aim was to improve the media. The first deviation from this aim occurred when it was realized that *in vitro* cultures provided a precise tool for the study of stimulatory and inhibitory substances. Not only synthetic, but also natural products of the most varied origins, were tested. Finally, the more recent investigations are oriented towards the analysis of cellular metabolism. This orientation is very promising and will be firmly established in a short time. If, finally, one compares the present review with that which White (297) published only four years ago, one can see the extraordinary increment in the studies done on the nutrition of tissue cultures.

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